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THE EFFECTS OF NITROGEN MUSTARD (METHYL BIS (β-CHLOROETHYL) AMINE HCl) ON THE IMMUNOLOGICAL RESPONSE OF THE RABBIT. II. THE EFFECTS OF NITROGEN MUSTARD ON THE BREAKDOWN OF A BACTERIAL ANTIGEN IN SPLENIC TISSUE

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IT has been shown by Spurr (1947), Bukantz *et al.* (1949), Schwab *et al.* (1950) and Green (1958) that when an antigen is injected following the administration of a course of nitrogen mustard, there is a delay in the appearance of antibody compared with normal animals.

Schwab *et al.* and Green have suggested that this delay may in part be due to a delay in the breakdown of antigen.

Topley (1930) using a " transfer " technique which involved the injection of splenic tissue from rabbits previously injected intravenously with *Salm. paratyphi* B into recipient rabbits showed that the spleen was concerned in the production of antibodies. He further demonstrated that within 24 hr. the antigen in the splenic tissue of the donor rabbits was broken down to the stage at which it was no longer antigenic to the recipients since they reacted to an intravenous injection of *Salm. paratyphi* B by producing a primary type of response.

The " transfer " type of experiment has recently been used by a number of workers (Wager and Chase, 1952 ; Hale and Stoner, 1953 ; Harris and Harris, 1954 ; Oakley, Warrack and Batty, 1954) and one of the findings on which all these workers agree is that in order that the transferred tissue or cells from immunized animals may carry on the production of antibody in the tissues of the recipients, the cells must not be processed in any way. Grinding or freezing and thawing the cells or suspending them in distilled water renders them inactive. It would appear that the investigation of the breakdown of antigen in splenic tissue might be facilitated by the use of tissue processed in the ways mentioned above.

The present investigation was undertaken to determine whether or not antigenic material could be demonstrated in the splenic tissues of normal and nitro-

gen mustard treated animals using a " transfer " type of technique with processed tissue.

The plan of the investigation was that rabbits were injected with a nitrogen mustard course followed by *Salm. typhi* H cells 24 hr. later. Thereafter the rabbits were killed at intervals and their splenic tissues after being processed by freezing and thawing and freeze drying were injected into recipient rabbits intraperitoneally. These recipients were examined for the production of antibody over a four weeks period and thereafter were injected intravenously with a small dose of *Salm. typhi* H cells and their agglutination titres estimated over a period of 3 weeks.

MATERIALS AND METHODS

The *Salm. typhi* H suspension and the nitrogen mustard solution were prepared as previously described (Green, 1958).

Schedule of treatment of donor animals

The rabbits were divided into 6 groups each group containing 2 donors and 2 recipients. The rabbits used weighed approximately 2 kg.

The following table (Table I) shows the treatment of the different groups of animals.

TABLE I.—*Treatment of Donor Rabbits as regards Administration of Nitrogen Mustard Course, Injection of Antigen and Time of Killing after Injection of Antigen*

		N.M. course*	Antigen 24 hr after N.M. course†	Time after antigen when donor killed			
				24 hr.	48 hr.	72 hr.	96 hr.
" Normal " animals	I	−	−	−	−	−	−
	II	−	+	+	−	−	−
" Conditioned " animals	III	+	+	+	−	−	−
	IV	+	+	−	+	−	−
	V	+	+	−	−	+	−
	VI	+	+	−	−	−	+

* N.M. course.—Four daily intravenous doses of 1 mg. methyl bis (β-chloroethyl) amine HCl/kg. body weight.

† Antigen.—1000 million *Salm. typhi* H cells/kg. body weight injected intravenously 24 hr. after the last dose of N.M. course.

Drying of splenic tissue

The donors were bled out under Nembutal anaesthesia and their spleens were removed and, after a small piece had been removed for histological fixation and staining, were cleaned of fat, weighed and ground in a Griffith's tube with sterile distilled water. The tissue was then frozen and thawed twice and thereafter dried from the frozen state over $CaCl_2$ for 4 days. The dried tissue was ground finely in an agate mortar and stored at 4°.

Rehydration and injection of splenic tissue

x mg. of dried splenic tissue were weighed and $\frac{x(K-1)}{1000}$ ml. sterile distilled water was added where K is the factor of $\frac{\text{wet}}{\text{dry}}$ weight of splenic tissue.

The dried splenic tissue took up the water rapidly at room temperature. Before injecting the rehydrated tissue into the recipients, normal saline was added to the tissue to bring the total volume to 2 ml.

The recipient rabbits were injected intraperitoneally and each recipient received the splenic tissue from one donor.

Intravenous injection of Salm. typhi *H and test bleeding of recipient rabbits*

The recipients were bled before the intraperitoneal injection of the splenic tissue and every 2 days thereafter for a period of 4 weeks. At the end of this period, the recipients were injected intravenously with 10 million *Salm. typhi* H cells and test bleeds were again taken every 2 days for a further 3 weeks.

The methods of test bleeding and performing agglutination tests are those previously described (Green, 1958).

Histological preparations

Paraffin sections of the splenic tissues were made and stained by Haematoxylin-Eosin and Unna-Pappenheim.

RESULTS

No agglutinins to *Salm. typhi* H were demonstrated within the 4 weeks following the intraperitoneal injection of splenic tissue in any of the groups.

Following the intravenous injection of 10 million *Salm. typhi* H cells in the recipients, 2 types of response were obtained as will be seen in Table II.

TABLE II.—*Agglutination Titres of the Recipients in the Different Groups Following the Intravenous Injection of* 10 *million* Salm. typhi *H.*

Also shown is the treatment of the donors in the different groups.

Days following 10 M *Salm. typhi* H i/v.	"Normal" groups				"Conditioned" groups							
	Group I (No antigen)		Group II (A : 24)		Group III (N : A : 24)		Group IV (N : A : 48)		Group V (N : A : 72)		Group VI (N : A : 96)	
1	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	100	50	25	25	0	0	0	0
5	25	50	100	100	800	800	400	400	200	100	200	100
7	100	200	400	100	1600	1600	800	800	400	400	400	400
9	200	400	400	200	1600	800	800	800	400	400	200	400
11	200	400	400	200	800	800	800	400	400	200	200	200
13	100	200	200	100	800	800	800	400	200	200	200	200
15	100	200	200	100	800	400	800	400	200	200	100	100
17	50	100	100	100	400	400	400	400	200	100	100	100
19	50	100	100	100	400	200	400	400	100	100	100	100
21	50	100	100	100	400	200	200	200	100	100	100	50

A = 1000 million *Salm. typhi* H cells intravenously/kg. body weight.
N = nitrogen mustard course.
24, 48, 72, 96 = animal killed 24 hr., 48 hr., 72 hr. and 96 hr. following *Salm. typhi* H.

The first type of response was shown by the recipient animals of the "normal" Groups I and II and of the "conditioned" Groups V and VI and was characterized by the appearance of agglutinins on the fifth day following the injection of *Salm. typhi* H intravenously and the maximum titres did not exceed 1/400. It will be noticed that the donors of Group I ("normal") had not been injected with *Salm. typhi* H before they were killed and therefore the recipients of their processed tissue must give a primary type of response on being injected with *Salm. typhi* H. Since the responses of the recipients of the "normal" Group II donors and of the "conditioned" Groups V and VI donors are similar to those of the recipients of Group I, these responses are also of the primary type though all the donors were injected with *Salm. typhi* H before being killed.

The second type of response was shown by the recipients of the "conditioned" Groups III and IV in which agglutinins were detected on the third day following

the intravenous injection of *Salm. typhi* H and the maximum titres were 1/1600 or 1/800. The " after " peak titres were also higher than those showing the first type of response.

It will be seen from these results that in the case of the recipients of the " conditioned " Groups III and IV the responses are different from those of the " normal " Groups I and II and the " conditioned " Groups V and VI in these respects :

(1) Earlier appearance of agglutinins (3rd day as against 5th day).
(2) Higher maximum titres (1/1600 or 1/800 as against 1/400 or 1/200).
(3) Maintenance of higher levels after peak titre.

These 3 features are characteristic of a secondary type of response to a bacterial antigen as has been shown by Topley (1930) and Burnet and Fenner (1949).

The histological preparations, made from the spleens of the donors, showed that there was a difference in the reactions in the splenic tissues between the " normal " and " conditioned " animals.

In the normal animals injected with antigen and killed 24 hr. later, the splenic pulp was full of polymorphonuclear leucocytes and a few polymorphonuclears had also infiltrated the Malpighian bodies. This histological feature was absent in all the donors which had received a course of nitrogen mustard before the injection of *Salm. typhi* H (*i.e.*, " conditioned " groups). In these conditioned donors there was a total absence of polymorphonuclear leucocytes in the pulp at 24 hr., 48 hr. and 72 hr. following the injection of antigen (Groups III, IV and V) and only an occasional polymorphonuclear leucocyte at 96 hr. (Group VI) and in all cases there were no polymorphonuclears in the shrunken Malpighian bodies.

DISCUSSION

The striking observation obtained in these experiments is the different responses of the recipients of the processed splenic tissues taken from variously treated donors to an intravenous injection of *Salm. typhi* H. In the case of the recipients of processed tissue from " normal " donors (Groups I and II) or " conditioned " donors killed 72 hr. or 96 hr. following the injection of *Salm. typhi* H the response is that of the primary type. The obtaining of a primary type of response is in agreement with Topley's findings and the absence of agglutinins following the injection of the processed splenic tissue confirms the findings of Wager and Chase (1953), Harris and Harris (1954) and Oakley *et al.* (1954) that the tissue must not be processed if the production of antibody from the transplant is to continue.

In the case of the recipients of processed tissue from the " conditioned " donors of Groups III and IV killed 24 hr. or 48 hr. following the injection of *Salm. typhi* H the response to an intravenous injection of *Salm. typhi* H was of the secondary type. The presence of a secondary type of response in these recipients indicates that at the time at which the donors were killed there was still some *Salm. typhi* H present in the splenic tissue in a form which was antigenic to the recipients.

This antigenic material was capable of stimulating the recipients so that they reacted on a subsequent inoculation of *Salm. typhi* H by producing a secondary type of response, although no agglutinins could be demonstrated following the intraperitoneal injection of the processed tissue. The continued presence of

antigenic material at 24 hr. or 48 hr. is an indication of a slowing down of the process of degradation of the antigen due presumably to the effects of the nitrogen mustard. Although this antigenic material appears to be present in the processed tissue of " conditioned " donors killed 24 hr. or 48 hr. following the antigen (Groups III and IV), it was not demonstrable in the " conditioned " donors killed 72 hr. or 96 hr. following the antigen (Groups V and VI).

The interpretation of the demonstrated delay in the breakdown of *Salm. typhi* H in the " conditioned " donors of Groups III and IV is admittedly difficult but the observation serves to focus attention on the wider issue of the possible significance of the polymorphonuclear leucocytes in the production of antibody. The correlation between the low polymorphonuclear count at the time of injection of *Salm. typhi* H and the delay in the production of agglutinins (Green, 1958) and the delay in the breakdown of antigen in nitrogen mustard treated animals associated with the absence of polymorphonuclear leucocytes in the splenic tissue of such animals suggests the possibility that the polymorphonuclear leucocyte plays a significant role in the process of antibody production. This evidence could be interpreted on the basis that the polymorphonuclear cell is responsible for the phagocytosis and initial breakdown of the antigen to a stage which the macrophage-plasma cell series system can process further with the ultimate production of antibodies. The indirect evidence of a delay in the breakdown of antigen in the present experiments is supported by the direct evidence of delay in the disappearance of bovine gamma globulin in nitrogen mustard treated animals reported by Schwab *et al.* (1950).

It could be argued that the correlation between the polymorphonuclear leucocyte and the production of antibody in nitrogen mustard treated animals is contemporaneous without being causal. Nitrogen mustard is a general protoplasmic poison and is not selective for polymorphonuclears so that its effect on antibody production may operate through its action on the macrophage-plasma cell series system. The evidence for the view that there is a slowing down of the phagocytic and digestive activities of the macrophages is conflicting. Taliaferro and Taliaferro (1954) using the effects of X-rays on the production of immune haemolysins were of the opinion that there is an interference with the localization and early degradation of antigen, while Fitch *et al.* (1954) using X-rays and *Salm. typhi* labelled with I^{131} as antigen produced evidence that there was no delay in localization and degradation of the antigenic material.

Although the question of the mechanism of the breakdown of antigen should at present remain an open one, it is obviously desirable that further experimentation on the possible significance of the polymorphonuclear leucocyte in this mechanism should be undertaken.

SUMMARY

When the processed splenic tissue from normal donor rabbits injected intravenously with *Salm. typhi* H and killed 24 hr. later is injected intraperitoneally into recipient rabbits, the recipients react to a subsequent injection of *Salm. typhi* H intravenously by producing a primary type of agglutination response.

When the processed splenic tissue is taken from nitrogen mustard treated donors, two types of response are obtained in the recipients of the splenic tissue depending on the time at which the donors were killed following the injection of *Salm. typhi* H.

When the nitrogen mustard treated donors were killed 24 hr. or 48 hr. following the injection of *Salm. typhi* H, a secondary type of response is obtained in the recipients of their processed tissue in response to an intravenous injection of *Salm. typhi* H. If, however, the nitrogen mustard treated donors are not killed until 72 hr. or 96 hr. following the injection of antigen, the type of response obtained in the recipients of their processed splenic tissue following an intravenous dose of *Salm. typhi* H is of the primary type.

In none of the groups of recipients were agglutinins demonstrable following the intraperitoneal injection of the splenic tissue and before the intravenous injection of *Salm. typhi* H.

Histologically the marked polymorphonuclear infiltration of the splenic pulp seen in the normal donors 24 hr. after the injection of the antigen is absent in the nitrogen mustard treated animals up to 96 hr. after the antigen.

The possible function of the polymorphonuclear leucocyte in the mechanism of antigen breakdown is discussed.

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STUDIES ON THE ANTIGENIC STRUCTURE OF *BRUCELLA SUIS* WITH THE AID OF THE AGAR GEL PRECIPITATION TECHNIQUE.

I. The Resistance of Antigens to Physical, Chemical and Enzymatic Treatments

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Investigations on the antigenic structure in the genus *Brucella* have led to different results. Wilson and Miles (1932) explained the serological differences between the strains of the three species *Br. abortus*, *Br. melitensis* and *Br. suis* by the quantitative distribution of two antigens, A and M, the A antigen being in excess in *Br. abortus* and *Br. suis*, the M antigen in *Br. melitensis*. Olitzki and Gurevitch (1933) explained the antigenic differences by the presence of two specific antigens, A, specific for *Br. abortus* and M, specific for *Br. melitensis*, with a non-specific common antigen, G. Renoux and Mahaffey (1955) proposed a more detailed antigenic scheme of the *Brucella* genus by which the S-strains of the three species contain the antigens A, M, Z, and r in different distributions. An ovine strain from New Zealand contains only Z and r, and the R-strains contain only r with or without Z. It seemed worthwhile, therefore, to re-examine the antigenic structure of the *Brucella* genus by the method described by Oudin (1946) and Ouchterlony (1948) in view of the clear results obtained by the latter method in the *Pasteurella* genus by Chen and Meyer (1955), Crumpton and David (1956), Davis (1956) and Burrows (1956).

Preliminary experiments of Olitzki and Sulitzeanu (1957) showed that as a result of the reaction between the sonic extract prepared from *Br. suis* and its antiserum prepared by immunization of rabbits with acetone-killed bacteria, 5 lines, at least, appeared in agar gel.

At the reaction of *Br. melitensis* with its antiserum there appeared 6 lines, partially confluent with undiluted antigen, which became distinct, although faint, at higher antigen dilutions; among them 5 strong lines were easily recognized.

In continuation of this work, Olitzki and Sulitzeanu (1957) employed a hyperimmune serum and regularly produced at least 6 precipitation lines and an additional diffuse line (7 or D). When a serum prepared with the aid of Freund's adjuvant was used, 3 sharp lines appeared in the region of line 7, which could, therefore, be assumed to be a complex of 3 components. An additional strong precipitation line (M) was obtained when the anti-*Br. suis* hyperimmune serum was set up against *Br. melitensis* antigen, although this line was hardly distinguishable in the reaction zone between *Br. suis* antigen and the corresponding immune serum. Thus at least 10 distinct lines could be differentiated.

Absorption of immune serum by whole bacteria killed by acetone removed lines 4, 5, and 6, but not lines 1, 2, and 3, indicating that these latter antigens are situated in deeper layers of the bacterium.

Bruce and Jones (1957) applied the agar gel precipitation method to a study of the antigenicity of *Br. melitensis*. The number of precipitin lines with sera of rabbits, goats, and cattle that had been infected either with *Br. abortus* or with *Br. melitensis* varied from one to three. A diffusible antigen could not be prepared, however, from *Br. abortus* and *Br. suis* cultures by methods which were effective in the case of *Br. melitensis*.

The purpose of the experiments described below was to distinguish the different antigens of *Br. suis* by physical and chemical procedures and to determine the conditions under which a maximal number of precipitation lines is produced and those under which the formation of these lines is inhibited.

EXPLANATION OF PLATES.

FIG. 1.—Effect of heating at 60° on the precipitability of a sonic extract.
Se = Immune serum.
E = Unheated extract.
E_{60} = Extract heated at 60° for 30 min.
All lines persisted. The figure shows mainly the thin lines 3–5 and the thick line 6.

FIG. 2.—Right side: Effect of heating at 100° on the precipitability of bacterial extracts. Left side: Influence of exposure to NaOH for 4 hr. on acetone-killed bacteria.
Se = Immune serum.
B = Acetone-killed bacteria.
E_{100} = Sonic extract heated at 100° for 2½ hr.
NaOH = Acetone-killed bacteria exposed to 0·05 N NaOH for 4 hr. at 20°.

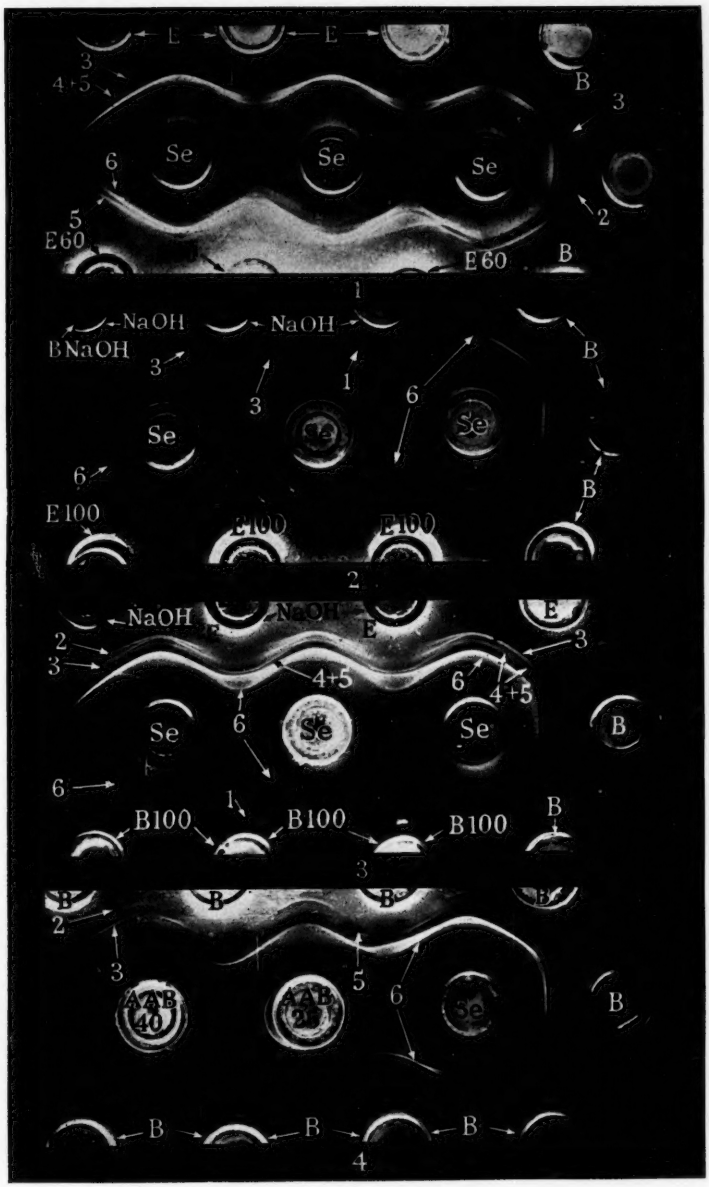
FIG. 3.—Right side: Influence of heating at 100° on acetone-killed bacteria. Left side: Influence of exposure to NaOH on a sonic extract.
B = Acetone-killed bacteria.
E = Sonic extract.
NaOH E = Sonic extract exposed to 0·05 N NaOH for 4 hr. at 20°.
B_{100} = Acetone-killed bacteria heated at 100° for 2½ hr.

FIG. 4.—Absorption with acetone-killed bacteria.
Se = Immune serum not absorbed.
AAB25 = Serum absorbed with 25 mg. of acetone-killed bacteria.
AAB40 = Serum absorbed with 40 mg. of acetone-killed bacteria.
B = Acetone-killed bacteria.

FIG. 5.—Absorption tests with two different antigens: (*a*) sonic extract heated at 100°, (*b*) acetone-killed bacteria exposed to NaOH.
B = Acetone-killed bacteria.
Se = Immune serum not absorbed.
A NaOH B = The same serum absorbed with bacteria treated with 0·05 N NaOH for 4 hr. at 20°.
A E_{100} = The same serum absorbed with extract heated at 100° for 2½ hr.

FIG. 6.—Absorption of immune serum with acetone-killed bacteria heated at 100°.
B = Acetone-killed bacteria.
Se = Immune serum, not absorbed.
AB_{100} 25 = Serum absorbed with 25 mg. acetone-killed bacteria heated at 100° for 2½ hr.
AB_{100} 40 = Serum absorbed with 40 mg. of the same antigen.

FIG. 7.—Absorption test with HCl-treated bacteria.
B = Acetone-killed bacteria.
Se = Immune serum not absorbed.
AB 0·1 N HCl = Serum absorbed with acetone-killed bacteria exposed to 0·1 NHCl for 24 hr. at 20°.



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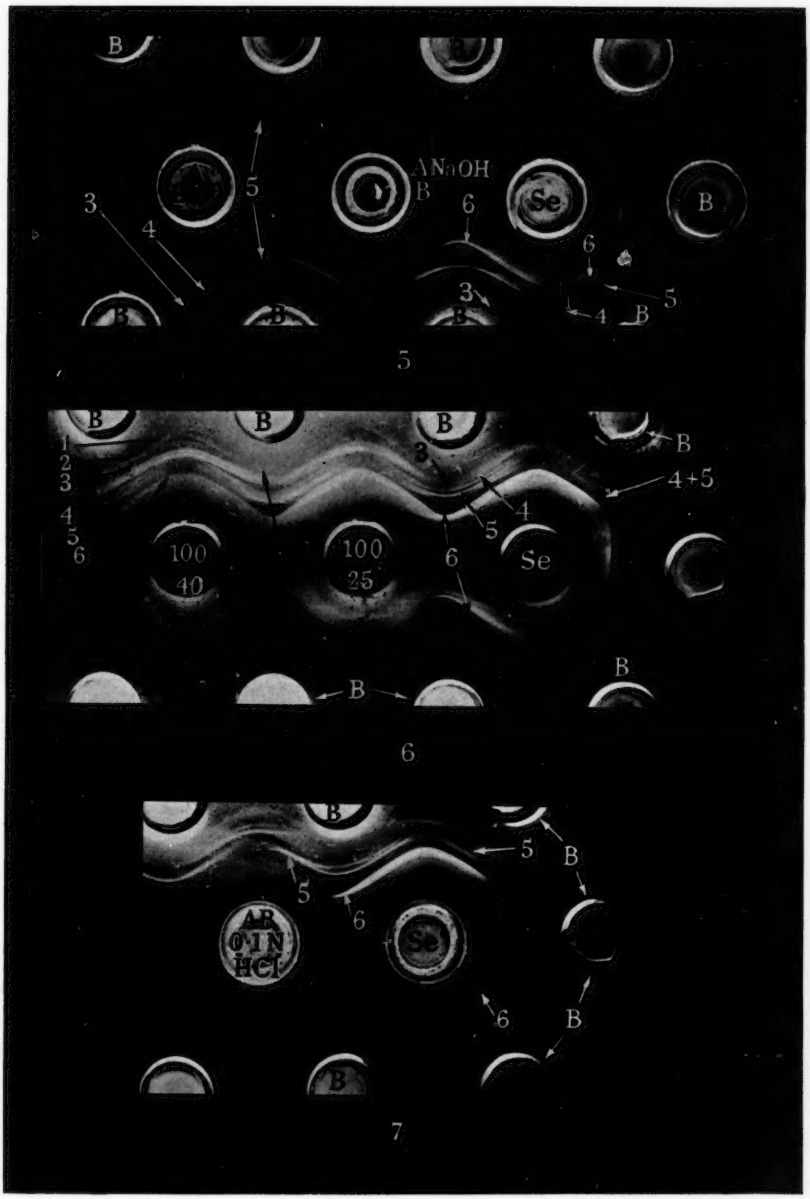

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MATERIALS AND METHODS

Antigen for serum preparation

The antigens were prepared from strain 39 of *Br. suis*. Bacteria were grown in Roux bottles on 10 per cent trypticase soy agar in nutrient agar, supplemented with 3 per cent glycerol and 1 ml./litre of 0·01 per cent thiamine solution. After 4 to 5 days' incubation at 37° the growth was washed off with a small volume of saline, the suspension poured into 3 volumes of cold acetone (−20°) and left to stand overnight. The next day the bacteria were washed 3 times with cold acetone and dried in a dessicator, *in vacuo*, over $CaCl_2$.

Preparation of immune serum

Rabbits were immunized by intravenous injections of acetone-dried bacteria, 3 times a week, starting with 0·05 mg. and increasing gradually to 1 mg. After 6–8 weeks, strongly precipitating immune sera were obtained. The injections were continued, however, throughout the period of bleeding, with occasional pauses of 2–3 weeks. Another group of rabbits received bacteria incorporated in Freund's adjuvant, prepared according to Salk and Laurent (1952). The adjuvant mixture contained 20 mg. bacteria per ml. and was given 3 times, 0·5 ml. intramuscularly and 0·5 ml. subcutaneously, at fortnightly intervals. Blood was obtained by cardiac puncture 2 weeks after the last injection.

Antigens for gel precipitation

The following antigens were employed:

(*a*) Acetone-dried bacteria prepared as described above.

(*b*) Sonic extracts prepared from acetone-killed organisms in a 9 kc Raytheon magnetostriction oscillator. The sonic extract was centrifuged to remove debris and the supernatant served as antigen. It was diluted to contain about 1·5 mg. protein per ml. and was stored in the refrigerator with merthiolate 1/10,000 as preservative.

(*c*) Extracts prepared as in (*b*) except that the bacteria were disintegrated in a tissue disintegrator (Mickle, Mill Works, Gomshall, Surrey). It was found necessary to subject the bacteria to 3 to 4 treatments, since the efficiency of the instrument in disrupting the organisms was low.

(*d*) Extracts from living bacteria were prepared by grinding with alumina powder according to McIlwain (1948).

Diffusion tests

The tests were performed in Petri dishes, in which a first layer of 10 ml. of one per cent agar in phosphate buffer (pH 7·4) was poured. Antibiotic testing cups were then placed in position and a second 10 ml. layer poured. Three to 5 drops of reagent were placed in the cups and the Petri dishes stored in a closed metal box at 37° for 4–10 days. Cotton-wool moistened with water served to prevent drying.

Resistance of the diffusable antigen to heating and chemical agents

The resistance of the 6 main antigens to heating at 60° for 30 min. and 100° for 2½ hr. was examined. The acetone-treated bacteria were, furthermore, exposed to 1·0 N and 0·1 N HCl for 24 hr. and to 0·05 N NaOH for 4 hr. at 20°. The resistance of the precipitating property and of the combining capacity of each antigen was tested. Since the disappearance of one of these properties from intact bacteria could be explained on the basis of partial or complete destruction or removal of the antigen, the supernatants were also tested. Sonic extracts from acetone-killed bacteria were tested in the same way as the intact bacteria.

Absorption of sera with bacteria and extracts

Bacteria.—The sera were diluted 1/1·5. This dilution gave the best differentiation of diffusion lines. Twenty-five mg. bacteria were used to absorb 1 ml. of diluted serum, this quantity being adequate to remove all agglutinins. After being subjected to the desired treatment the suspension containing 25 mg. bacteria was centrifuged, 1 ml. of serum was added to the sediment and thoroughly mixed. The tube was placed in a 37° water bath for

2 hr. and then in the 6° cold room for 18 hr. The bacteria were removed by further centrifugation and the serum was ready for testing.

Extracts.—To 1 ml. of undiluted serum 0·5 ml. of the appropriate extract were added. The mixture was kept at 37° for ½ hr. and overnight at 6° and then centrifuged to remove the precipitate.

Lyophilization procedure

A dense suspension of bacteria was prepared in Trypticase soy broth divided into ampoules, frozen in CO_2-alcohol mixture and dried in a centrifuge freeze drying apparatus (W. Edwards & Co., London). The ampoules were sealed *in vacuo*.

Treatment of sonic extracts with proteolytic enzymes

Trypsin.—Five ml. of sonic extract, containing about 40 mg. protein, were brought to pH 8·3 with NaOH solution. Then 0·5 mg. trypsin and 0·25 ml. toluol were added and the mixture was incubated at 37°. After 24 hr. the pH, which had dropped below 7, was re-adjusted to 8·3. Four additional days of incubation did not cause any further pH changes.

Papain.—Ten mg. papain and 8 mg. cysteine were added to 5 ml. sonic extract. The pH was adjusted to 7·0 and the mixture placed in the water bath, at 52°, for 2 hr.

Pepsin.—The following mixture was prepared in a final volume of 5 ml.: Sonic extract approx. 40 mg. protein), pepsin 10 mg., NaCl 40 mg., N. HCl 0·3 ml.

The mixture was placed in a water bath at 52° for 4 hr., with occasional shaking. It was then centrifuged and the supernatant neutralized and used in the test.

Controls with the same reagents as the test but without the corresponding enzyme were run at the same time.

Lysozyme.—To 40 mg. bacteria suspended in 20 ml. M/15 phosphate buffer (pH 7) 2 mg. lysozyme were added. The mixture was incubated for 3 hr. at 37°. A control suspension of identical composition, but with the enzyme omitted, was similarly treated. When removed from the water bath, the lysozyme-treated suspension was markedly opalescent, showing that a fair percentage of the bacteria had been disintegrated. This was confirmed by microscopical examination of Gram-stained slides, which showed large masses of Gram-negative, amorphous material, presumably cell debris.

The control and test suspensions were centrifuged and the sediments resuspended in the original saline volume. Sediments and supernatant fluids were then placed in cups in the usual way.

RESULTS

Experiments reported elsewhere have shown that 5–6 lines can be obtained with a *Br. suis* antiserum, when set up against acetone-killed bacteria or their sonic extracts in agar plates. Acetone-killed bacteria could not be replaced by living organisms, since the latter only produce a very weak line under similar conditions (antigen 6).

The effect of some of the treatments on the precipitation pattern of *Br. suis* antigens is represented in Fig. 1–8. These figures do not show, however, all the lines which were visible to the naked eye.

Fig. 1 shows the effect of heating the ultrasonic extract of acetone-killed bacteria at 60° for 30 min. All 6 lines persisted, lines 3–6 are visible in the photograph.

Fig. 2 (right side) shows the effect of heating the sonic extract of acetone-killed bacteria at 100° for 2½ hr. Line 6 only persisted as a broad diffuse line. On the left side the influence of NaOH treatment of intact bacteria is demonstrated: only lines 1 and 3 persisted, the other disappeared. This result was obtained with only one hyperimmune serum; the others showed no lines under the same conditions.

Further experiments indicated that this disappearance was due to extraction of the antigens by NaOH from the bacterial bodies. This was proved by the precipitating potency of the supernatant fluid after NaOH treatment of the intact bacteria and by the resistance of the precipitating potency of sonic extracts to NaOH.

Fig. 3 (right side) demonstrates the effect of heating at 100° on intact acetone-killed bacteria. Only lines 1, 3 and 6 persist faintly. The disappearance of the other lines was to some extent due to the destruction of the antigens. The result of this experiment supplements that illustrated in Fig. 2 proving the relative stability of antigen 6.

The left side of Fig. 3 shows that all 6 antigens were preserved when sonic extracts were treated with NaOH.

Fig. 4 demonstrated an absorption experiment with acetone-killed bacteria. After addition of 25 mg. bacterial substance, lines 4 and 5 disappeared, after addition of 40 mg., line 6 became weak while lines 1–3 persisted. In other immune sera, lines 4, 5 and 6 disappeared completely after absorption with acetone-killed bacteria.

Fig. 5 shows that the precipitating power of an immune serum is still preserved after its absorption by NaOH-treated bacteria. NaOH treatment causes the extraction of all antigens and, therefore, bacteria thus treated, cannot absorb any antibodies.

On the other hand, line 6 has disappeared after absorption with a sonic extract heated at 100°, showing the stability of the combining power as well as the precipitability of antigen 6.

Fig. 6 shows an absorption experiment with bacteria heated for $2\frac{1}{2}$ hr. at 100°, using quantities of 25 and 40 mg. of bacterial substance. All 6 lines were well preserved. The preservation of lines 1, 2, 3 was due to the fact that the antibodies cannot be absorbed even by untreated bacteria as already explained. Lines 4, 5, 6, however, were preserved because of the destruction of the corresponding antigens by the heat treatment.

In Fig. 7 an absorption experiment with 0·1 N HCl-treated bacteria is seen. Line 6 disappeared and 5 became weaker, due mainly to the extraction of these antigens from the bacterial bodies.

In addition to the experiments illustrated in Fig. 1–7, many other experiments were carried out with the same methods. The results are presented in Tables I–IV.

Table I shows the precipitating properties of intact bacteria after different treatments.

Living bacteria showed only line 6 and that, weakly. After treatment with acetone all 6 precipitation lines appeared. The antigens seemed to be fixed loosely to the bacterial bodies since after suspension and centrifugation in saline they appeared in the supernatant fluids, while the bacterial sediment showed after 2 washings an impaired capability to produce precipitation lines. When acetone-killed bacteria were suspended in saline and heated at 60° for $\frac{1}{2}$ hr. the heated suspension still produced all 6 precipitation lines. However, centrifugation and separate examination of the supernatants and the bacterial sediment proved that almost all antigens were easily liberated from the bacterial bodies and appeared in the supernatant fluids after heating, while the bacterial sediment after heating and 2 washings produced only lines 3 and 6.

TABLE I.—*Precipitating Properties of Intact Bacteria (Non-disintegrated) and Their Supernatant Fluids after Different Treatments*

Treatment of bacteria	Lines obtained					
	1	2	3	4	5	6
Living, untreated	−	−	−	−	−	±
Acetone-killed, not washed	+	+	+	+	+	+
Acetone-killed and centrifuged twice :						
(1) Supernatant—1st centrifugation	+	+	+	+	+	+
(2) „ 2nd „	+	+	+	+	+	+
(3) Bacterial sediment, after 2nd centrifugation	±	±	±	±	±	±
Acetone-killed and heated 0·5 hr. at 60° :						
(1) Not washed	+	+	+	+	+	+
(2) Supernatant—1st centrifugation	+	+	+	+	+	+
(3) „ 2nd „	−	−	+	±	−	+
(4) Bacterial sediment after 2nd centrifugation	−	−	+	−	−	+
Acetone-killed and heated 2½ hr. at 100° :						
(1) Not washed	V*	−	+	−	−	±
(2) Supernatant—1st centrifugation	−	−	+	−	−	±
(3) „ 2nd „	−	−	−	−	−	−
(4) Bacterial sediment after 2nd centrifugation	−	−	+	−	−	±
Acetone-killed and heated with 1·0 N HCl 24 hr. at 20° :						
(1) Supernatant—1st centrifugation	−	−	−	−	−	±
(2) „ 2nd „	−	−	−	−	−	±
(3) Bacterial sediment after 2nd centrifugation	−	−	−	−	−	−
Acetone-killed and treated with 0·1 N HCl 24 hr. at 20° :						
(1) Supernatant—1st centrifugation	−	−	+	+	+	+
(2) „ 2nd „	−	−	+	−	−	+
(3) Bacterial sediment after 2nd centrifugation	−	−	−	−	−	±
Acetone-killed and treated with 0·05 N NaOH 4 hr. at 20° :						
(1) Supernatant—1st centrifugation	+	+	+	+	+	+
(2) „ 2nd „	−	−	+	−	−	+
(3) Bacterial sediment after 2nd centrifugation	V*	−	V*	−	−	−

* V = Variable.

After heating at 100° antigens 1, 2, 4 and 5 were destroyed. The supernatant obtained after centrifugation, and the bacterial sediment obtained after an additional washing in saline contained only antigens 3 and 6, while the supernatant after the second centrifugation was completely devoid of any antigenic power.

Acetone-killed bacteria treated with 1·0 N HCl for 20 hr. did not produce any line, while in the supernatant fluids antigen 6 still persisted.

After treatment with 0·1 N HCl for 20 hr. antigen 6 still persisted in the washed bacteria, while after the centrifugation antigens 3, 4, 5, and 6 persisted. After a second re-suspension and centrifugation antigens 3 and 6 appeared in the supernatant fluid proving their relative stability to 0·1 N HCl.

Acetone-killed bacteria treated with 0·05 N NaOH and washed contained sometimes traces of antigens 1 and 3. After centrifugation the supernatant fluid still contained all 6 antigens proving that NaOH treatment promoted the release of the antigens from the bacteria, but did not destroy them. After a second re-suspension in saline and centrifugation antigens 3 and 6 still appeared in the supernatant fluid.

All these experiments proved that heating at 60° and treatment with 0·05 N NaOH furthered the release of antigens from the bacteria without destroying them, while heating at 100° and treatment with HCl destroyed most of the anti-

gens. Antigens 3 and 6 showed a relatively higher stability against these treatments than the other ones.

Table II shows that extracts obtained from living bacteria by the method of McIlwain (1948) or from acetone-killed bacteria by the oscillator contain all 6

TABLE II.—*Precipitating Properties of Extracts Obtained by Disintegration of Bacteria*

Treatment of bacteria		Precipitation lines					
Extracts derived from	Treatment after disintegration	1	2	3	4	5	6
Living bacteria (A)	None	+	+	+	+	+	+
Acetone-killed bacteria (O)	"	+	+	+	+	+	+
" " "	Heated 0·5 hr. at 60°	+	+	+	+	+	+
" " "	" 2·5 " " 100°	−	−	−	−	−	±
" " "	Treated with N HCl 24 hr. at 20°	−	−	−	−	−	−
" " "	" " 0·1 N HCl 24 hr. at 20°	−	−	−	−	−	−
" " "	" " 0·05 N NaOH 4 hr. at 20°	+	+	+	+	+	+

A = Disintegration by Alumina powder.
O = Disintegration by Oscillator.

antigens. Heating at 60° for ½ hr. or treatment with 0·05 N NaOH does not reduce the number of precipitation lines, while heating at 100° for 2½ hr. leaves only antigen 6. On the other hand, the treatment with HCl for 20 hr. removes all 6 lines.

Table III shows the combining power of non-disintegrated bacteria. Living bacteria were able to remove all agglutinins from an immune serum, while the

TABLE III.—*Combining Power of Non-disintegrated Bacteria*

Absorbing antigen	Absorbed serum: Precipitation lines 1	2	3	4	5	6	Agglutination
Living bacteria	+	+	+	+	+	+	0
Acetone-killed bacteria	+	+	+	−	−	−	0
" and heated 0·5 hr. at 60°	+	+ or ±	+	± −	± −	± −	0
" " " 2·5 " " 100°	+	+ or ±	+	± +	± +	± +	0
" " treated with N HCl 24 hr. at 20°	+	+	+	+	+	−	0
" " " with 0·1 N HCl 24 hr. at 20°	+	+	+	+	±	−	0
" " " with 0·005 N NaOH 4 hr. at 20°	+	+	+	+	+	+	0
Control: Non-absorbed serum	+	+	+	+	+	+	640

Agar gel precipitation and agglutination of non-absorbed and of absorbed sera was carried out with acetone-killed bacteria.

6 precipitation lines persisted. Acetone-killed bacteria both untreated and treated at 60° for ½ hr. removed antibodies corresponding to antigens 4–6, while those corresponding to antigens 1–3 persisted. This result indicates that while the former were able to come in contact with their corresponding antigens, the latter were unable to do so, probably due to the location of these antigens in the deeper layers of the cell. Treatment with 0·05 N NaOH and heating at 100° for 2½ hr. deprived the bacteria of combining power for all 6 antigens, while after treatment with HCl their combining power for the anti-6 immune body persisted.

TALBE IV.—*Combining Power of Extracts Obtained by Disintegration of Bacteria*

Extracts derived from	Treatment after disintegration	Precipitation lines of absorbed serum 1	2	3	4	5	6	Agglutination
Living bacteria	None	−	−	−	−	−	−	Not done
Acetone-killed bacteria	„	±	−	−	−	−	−	120
„ „	Heated 0·5 hr. at 60°	±	−	−	−	±	±	320
„ „	„ 2·5 „ „ 100°	+	+	+	+	+	±	40
„ „	Treated with N HCl 24 hr. at 20°	+	+	+	+	+	+	80
„ „	„ „ 0·1 N HCl 24 hr. at 20°	+	+	+	+	+	+	160
„ „	„ „ 0·05 N NaOH 4 hr. at 20°	V	V	V	V	V	V	160
Control: Non-absorbed serum	. . . . .	+	+	+	+	+	+	640

V = Results variable in different immune sera.

Agar gel precipitation and agglutination of non-absorbed and of absorbed sera was carried out with acetone-killed bacteria.

Table IV shows the combining power of extracts obtained by disintegration of bacteria. All extracts derived from living or acetone-killed bacteria were able to remove all 6 corresponding antibodies. Heating of the extract at 60° partially destroyed its combining power for anti-1, 5, and 6 immune bodies, while heating at 100° completely destroyed the combining power of antigens 1—5 and, incompletely, antigen 6. Treatment with HCl destroyed the combining power for all 6 antigens, and treatment with 0·05 N NaOH gave variable results with different immune sera.

Lyophilized living bacteria gave the same lines as acetone-killed bacteria. This was due to the fact, that about 80 per cent of the organisms were non-viable as determined by the plate counts. Lyophilization of acetone-killed bacteria produced no changes in their precipitation pattern.

Treatment of sonic extracts with trypsin and papain destroyed antigen 4. The pepsin treatment was complicated by the low pH used, as this alone was sufficient to inactivate antigens 4 and 5, although antigens 3 and 6 were preserved. Pepsin treatment caused inactivation of all antigens.

The disruption of the cells with lysozyme seemed to have no effect on the antigens themselves. The sediments of both test and control suspensions gave a few weak lines, showing that the antigens had been extracted. The supernatants gave practically all the lines, but these were stronger with the supernatant of the enzyme-treated bacteria. It was concluded that by breaking up the cell walls, the enzyme had caused a more efficient release of the antigens.

SUMMARY

Precipitating power.—The antigenic structure of *Brucella* has been studied by the agar gel precipitation technique using whole bacteria and bacterial extracts treated in different ways. Intact living bacteria put up against hyperimmune serum did not release antigens other than traces of antigen 6; acetone-killed and dried bacteria released all 6 antigens. The same results were obtained after heating at 60° for 30 min. After heating at 100° for 2½ hr. antigens 1, 3 and 6 were still released and after treatment with N NaOH no antigen was released into the agar gel.

Exposure of the sonic extracts to 60° and 0·05 N NaOH showed the stability of all 6 antigens to this procedure. Exposure to HCl damaged the precipitability of all 6 antigens, while heating at 100° impaired the precipitability of antigens 1–5, antigen 6 being only slightly affected.

Combining power.—Living bacteria did not absorb precipitating antibodies, while agglutinins were wholly absorbed.

Acetone-treated bacteria removed from immune serum only the antibodies corresponding to antigens 4–6 showing that these antigens readily come in contact with their specific antibodies, while antigens 1–3 seemed to be placed in deeper layers of the bacteria. Acetone-killed bacteria heated at 60° behaved in the same manner as those not heated.

Sonic extracts of living and acetone-killed bacteria absorbed all 6 antibodies, while after heating at 60° the combining capacity of antigens 1, 5 and 6 was somewhat reduced.

Heating at 100° and treatment with HCl destroyed the combining power of antigens 1–5 in sonic extracts. The combining power of antigen 6 showed relative stability; it resisted HCl treatment in intact bacteria, and, partially, heating at 100° in sonic extracts.

Treatment with 0·05 N NaOH removed all antigens from the bacterial bodies. The combining power of several non-identified antigens in the sonic extracts was destroyed.

Living bacteria subjected to lyophilization released all antigens into the agar gel, but 80 per cent were dead as determined by plate counts.

Antigen 4 was destroyed by trypsin and papain treatment. Pepsin inactivated all antigens. Lysozyme only increased the efficiency of the release of antigens from intact bacteria.

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ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION

IV. ACTIVABLE PERMEABILITY FACTOR AND ITS INHIBITOR IN THE SERUM OF THE RAT AND THE RABBIT

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A FACTOR increasing the permeability of small blood vessels (PF) is present in the serum of the guinea-pig as an inactive precursor, pro-PF. The serum also contains an inhibitor of PF (IPF), and the two together constitute a pro-PF/IPF system which is present not only in serum (Miles and Wilhelm, 1955; Wilhelm, Miles and Mackay, 1955; Wilhelm, Mill and Miles, 1957) but also in the lymph and intercellular fluid of the guinea-pig (Miles and Wilhelm, 1958). A brief survey of the permeability-increasing properties of a number of sera—man, rat, rabbit, cat, dog, horse, ox and sheep (Wilhelm *et al.*, 1955)—suggested that the pro-PF/IPF system was a general feature of mammalian blood. However, these sera were tested in the guinea-pig, so that the observed increase in vascular permeability might have been due to species incompatibility of guinea-pig tissues and the "foreign" constituents of the sera. We have accordingly investigated the system in detail in the rat and rabbit—laboratory animals that could be readily tested with preparations of homologous serum.

The investigation revealed a pro-PF/IPF system in rat and rabbit blood. Rat PF, like that of the guinea-pig, is activable by simple dilution, and appears to be an α-globulin; the IPF occurs in α_1-globulin and albumin fractions. The rabbit PF is not activable by simple dilution, and occurs in serum preparations containing both α- and β-globulins; rabbit IPF is associated with the proteins migrating electrophoretically in the albumin zone.

MATERIALS AND METHODS

Sera.—These were harvested either from the clot or after immediate centrifugation of defibrinated blood and were fractionated by the method of Kekwick and Mackay (1954).

It should be emphasized that, unless otherwise stated, all tests of serum preparations and serum fractions were made in animals of the *homologous* species.

Drugs.—Histamine was used as the acid phosphate (British Drug Houses), and 5-hydroxytryptamine (5-HT) as the creatinine sulphate (Upjohn Co., Kalamazoo, U.S.A.); the weights of each are cited as base. Compound 48/80 was supplied by the Wellcome Research Laboratories, Tuckahoe, U.S.A.; leukotaxine by Dr. W. G. Spector, University College Hospital Medical School, London; mepyramine maleate by May and Baker Ltd.; lysergic acid diethylamide (LSD) by Sandoz Ltd., London; and soya bean trypsin inhibitor (SBTI) and pancreatic trypsin inhibitor (PTI) by Worthington Biochemical Sales Co., New Jersey, U.S.A. Ovomucoid trypsin inhibitor was prepared according to Lineweaver and Murray (1947).

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Measurement of permeability effects.—As before, permeability-increasing potency was measured *in vivo* in terms of the diameter of the areas of exudation of circulating pontamine blue induced by intracutaneous injection of the substance under test (Miles and Wilhelm, 1955). The peculiarities of the skin of the trunk of the rat and rabbit entailed some modification of the technique of intracutaneous testing devised for the guinea-pig.

Rats.—Albino rats weighing 220–320 g. were tied by their feet to a board for depilation and for intravenous and intracutaneous injection. They were depilated and given 60 mg. pontamine blue per kg. body weight, as a 2·5 per cent solution in 0·6 per cent saline, into a vein on the inner aspect of the upper part of the thigh. Four antero-posterior rows of 4–5 intracutaneous injections were made in the skin, 3 cm. on each side of the dorsal midline, in an area extending from a point 2·5 cm. behind the posterior border of the scapula to the level of the lumbo-sacral junction. The area was mapped, and the injections made, in the moderately extended animal. Intracutaneous injections can be made quickly in the region of the lumbo-sacral junction, but not in the tougher skin of the upper thoracic region; accordingly, since smaller blebs are produced by quicker injection, it is important to give all injections at the same slow rate. When this precaution is taken, the skin of the test area is homogeneous in its PF responses.

Thirty min. after the injections, the rats were killed and their lesions measured. Surface measurements were usually satisfactory, but in definitive experiments the results were checked by measurements of the lesions on the under surface of the skin after reflection. The under-surface readings were consistently 3–4 mm. larger than those on the upper surface, but the slopes of the dosage-response lines were similar. In some of the inhibition tests, where the upper-surface lesion diameters were too near to that of traumatic blueing to be significant, under-surface readings were used for the assays. Batches of 4 rats were tested in each experiment; each recorded lesion-diameter is the mean of at least 4 lesions each in one rat, and sometimes of as many as 16 lesions, 4 in each rat.

The volume injected was 0·1 ml. With very sharp, short-bevel, No. 26 gauge needles a small moderately pale area of traumatic blueing 1–4 mm. in diameter develops at the centre of the bleb when 0·1 ml. saline is injected. In some animals the saline induced slightly larger lesions, but in all tests with batches of 4 rats the mean lesion diameter was less than 4 mm. When Locke's solution was used in place of saline, the traumatic blueing never exceeded 3 mm. in diameter; but in all cases we have regarded only those lesions whose diameter exceeds 4 mm. as indicating the presence of a permeability factor.

In terms of lesion-diameters measured either on the upper or the subcutaneous aspect of the skin, our results were at first inconsistent, owing to the acute peripheral vasomotor instability of partly-tamed laboratory stock during the handling necessary for depilation, intravenous and intracutaneous injection. Anaesthesia of a degree sufficient to suppress this instability was impracticable, because it depressed the cutaneous circulation and greatly diminished the PF response (cf. Miles and Miles, 1952). Good and consistent results were obtained with thoroughly tamed animals, habituated to frequent handling, especially before their weaning. The best results were obtained with non-moulting rats of a large, rapidly growing strain, which had reached the optimal weight of 230–280 g., a stage at which their skins had not yet acquired adult toughness.

Rabbits.—Non-moulting (see Whiteley, 1956) New Zealand Red rabbits heavier than 2·5 kg. body weight were used. Depilatories were not used because they readily "burnt" the skin. The skin of the trunk was closely clipped 1–2 days previously and the rabbits kept warm until tested. They received 60 mg. pontamine blue per kg. body weight as a 5 per cent solution in 0·425 per cent saline in a marginal ear vein.

The PF response of rabbit skin is not homogeneous. The skin of the back is preferable to that of the belly. On the back, the response is maximum in the region of the lumbo-sacral junction and declines as the upper thorax is approached. To counter the heterogeneous response, the preparations under test were partly randomized over one side of the back in a pattern consisting of 3 rows of 8 injection sites parallel to and about 1·5, 3 and 4·5 cm. from the spine, beginning in the moderately extended animal immediately behind the posterior rib and extending towards the tail for about 14 cm. On the other flank duplicate injections of the same preparations were made according to the scheme in Table I. This arrangement ensures *e.g.* that the duplicate of an injection in the left outside row near the tail (No. 8 in Table I) is made in the right inside row near the head, and so forth.

In all tests the lesions were measured after 30–40 min. and in most tests after 2 and 4 hr. The test area was wiped with a pad of cotton-wool soaked in liquid paraffin, which made the

TABLE I.—*Relation of Intracutaneous Sites used in Rabbit Assays for Duplicating 24 Partly Randomized Injections in the Right and Left Dorsum of the Trunk, to Minimize the Effect of the Lower Sensitivity to Permeability Factors in the Upper Thoracic, as Compared with the Lumbo-Sacral Region.*

Head

1	9	17		8	16	24
2	10	18		7	15	23
3	11	19		6	14	22
4	12	20	Midline	5	13	21
5	13	21		4	12	20
6	14	22		3	11	19
7	15	23		2	10	18
8	16	24		1	9	17

skin appear as clean as when depilated. Each recorded lesion-diameter is the mean of at least 4 lesions, two in each of 2 rabbits, and often of 8 lesions in 4 rabbits. Saline injections did not induce lesions greater than 2·5 mm. in diameter.

PF standards in assay.—As in the guinea-pig, the mean lesion-diameters of the reactions induced by 0·1 ml. volumes of serum PF preparations are linear with respect to log. dose in the rat and rabbit, within the range 4·0–9·0 mm. For histamine the range is 4·0–11·0 mm. in the rabbit, but in the rat the slope becomes steeper below the 5 mm. level and reliable responses were obtained only within the range 6·0–9·0 mm. The slope of the dosage-response lines for serum PF and for histamine in all three animals was of the order of 3·5; so that it was possible, by the intracutaneous technique to assay any one of these substances in terms of the other. The guinea-pig serum PF was active in all three species. Since we had most experience in maintaining high-potency and stable preparations of this material, and because the linearity of the histamine dosage-response was restricted in the rat, we used guinea-pig serum fractions as PF standards in the rat assays, and both histamine and the guinea-pig preparations—carefully assayed against each other—in the rabbit assays. Potencies in the appropriate tables are expressed in terms of guinea-pig PF (Batch 33) = 100.

The "Effective Blueing Dose" (EBD).—In certain circumstances it is convenient to use the effective blueing dose (EBD) as a measure of PF potency. This we originally defined (Miles and Wilhelm, 1955) for the guinea-pig as the amount of a PF in 0·1 ml. producing a 6 mm. lesion. For reasons we discuss below (p. 243) we now amend the definition as follows. One EBD is the amount of a permeability factor which, in the standard injection volume of 0·1 ml. on the average induces, in the skin of a blued animal *of a stated species*, a lesion that at the maximum development of the area of increased permeability is 6 mm. in diameter. For example, the species difference is such that 1 mg. histamine contains 1400 rat EBD and 37,000 rabbit EBD (Sparrow and Wilhelm, 1957). As an approximate comparative measure of potency the use of the EBD is justified by the similarity of the slopes of the linear dosage-responses to the various permeability factors tested in the various species of animal we have used. The slope of the lesion-diameter in mm. against log. dose in the guinea-pig and rat fluctuates between 3 and 4, and in the rabbit between 3 and 5. The usual values lie between 3·5 and 4·0.

Estimation of the effects of inhibitors.—Inhibitors of PF were either injected systemically and the PF tested locally in the skin, or were injected intracutaneously mixed with the PF. The effect of various inhibitors and destructive treatments were usually estimated directly from the position of the dosage-response lines at levels where their slopes were parallel, as *e.g.*, in Fig. 3. When a single concentration of a PF was tested (as, *e.g.*, in Fig. 4), the change in potency was calculated from the potency ratio antilog. $d : b$ where d is the difference of mean diameter of the lesions induced by the control and treated preparations, and b the median value for the slope, which was assumed to be 3·0 in rat, and 4·0 in rabbit assays.

Unless otherwise stated, the concentrations of substances in mixtures are given as final concentrations. All preparations of serum and serum fractions, of other permeability factors, *e.g.*, histamine, and of inhibitors were diluted in 0·85 per cent saline or Locke's solution.

Plasmin activity.—The plasmin activity of serum PF preparations was assayed by the digestion of 0·5 per cent casein. The mixtures of PF and casein in 0·05 M veronal buffer, pH 7·6, were incubated for 1 hr. at 37°. One ml. samples taken at 0 and 1 hr. were added to

1 ml. 0·6 M trichloracetic acid and the degree of proteolysis was estimated from the amount of tyrosine in the supernatant fluid, by a modification of the Folin assay (Lowry, Rosebrough, Farr and Randall, 1951), in terms of the amount of Folin-reactive material.

RESULTS

Rat Serum

A native PF (PF/Nat) and a PF activable by dilution (PF/Dil)

Rat serum displays a dilution effect broadly similar to that of guinea-pig serum (Miles and Wilhelm, 1955). That is, undiluted serum either has very little PF activity or induces a lesion 3–5 mm. in diameter and of a pale greenish-blue colour quite unlike the frank blue exudate induced by the PF activated by dilution of rat serum (PF/Dil). Freshly defibrinated sera, tested within 30 min. of bleeding, tend to contain much less of this native PF than sera harvested from a clot 1–2 hr. old. Nevertheless, substantial greenish-blue lesions are induced *on the under surface* even by fresh sera that induce no blueing on the upper surface of the skin. Sera a few hours old induce this permeability effect much more strongly on the upper surface as well. The PF responsible may therefore result from a rapid *in vitro* degenerative change of the serum and be analogous with the PF/Age that develops in guinea-pig serum when held for 24 hr. or more at room temperature. Like guinea-pig PF/Age this low potency native PF (PF/Nat) is insusceptible to mepyramine maleate, to serum IPF, and to soya bean trypsin inhibitor (SBTI).

The PF/Nat is fully matured in rat serum 4 hr. old; and here one EBD of PF/Nat occurs in 1/15 dilutions; *i.e.*, one ml. serum contains 150 EBD of PF/Nat. Since this represents about 5 per cent of the total (p. 234) permeability-increasing substances isolable from rat serum, PF/Nat was not further investigated.

A transiently appearing PF/Dil can usually, but not consistently, be elicited in fresh rat serum. Our best results are exemplified in Fig. 1. There is little activity 1–2 min. after dilution in the range 1/2–1/3645. After 13–14 min. at room temperature, a PF/Dil appears in the 1/5–1/15 dilutions, shifts to the 1/45–1/135 range after 1 hr., and disappears after 4 hr.

It is not possible to estimate directly the number of EBD of PF/Dil maximally activated in rat serum, because the PF/Dil peak is usually below the 6 mm. level by the time it has moved into the higher serum dilutions. By rough extrapolation of the declining curve of PF activity in 1/100–1/1000 dilutions that are 1 hr. old, it appears that 1 ml. serum contains some 500 EBD activable PF/Dil.

In terms of a pro-PF/IPF system like that we postulated for guinea-pig serum, the changes summarized in the 4 curves of Fig. 1 represent a rapid activation of a relatively small amount of pro-PF, and a subsequent inactivation by a native IPF whose action within a few hours is more effective than that of guinea-pig IPF on guinea-pig PF/Dil (Wilhelm *et al.*, 1955, 1957). There is however a technical difficulty in demonstrating the action of IPF on rat PF/Dil. Rat serum contains a substantial amount of a PF (PF/G2) and an IPF isolable by fractionation (see below), and the IPF preparation (fraction G1S/P) inhibits the PF/G2 (p. 240). However, this IPF fraction, like the corresponding fraction in guinea-pig serum, acts slowly so that we have been unable in rat assays to demonstrate that it has any action on PF/Dil, owing to the rapid disappearance of PF/Dil from dilutions of serum (Fig. 1). Nevertheless, rat PF/Dil is inhibited by guinea-pig IPF when the PF/IPF mixtures are tested in guinea-pig skin, suggesting that it

is analogous with guinea-pig PF/Dil. That inhibition is demonstrable in these circumstances, in spite of the instability of PF/Dil in diluted rat serum, is due to the greater potency of preparations of guinea-pig IPF compared with those of rat IPF, and the greater responsiveness of guinea-pig skin compared with rat skin to rat PF/Dil.

Rat PF/Dil differs from guinea-pig PF/Dil in one important respect. It is susceptible to local mepyramine maleate, 162 μg./ml. In contrast, neither PF/Dil nor PF/G2 in the guinea-pig is susceptible to mepyramine, and rat PF/G2 is only moderately so. In another important respect however the parallelism of rat and guinea-pig PF preparations is complete; like guinea-pig PF/Dil and PF/G2, rat PF/Dil and PF/G2 are susceptible to SBTI, 100 μg./ml.

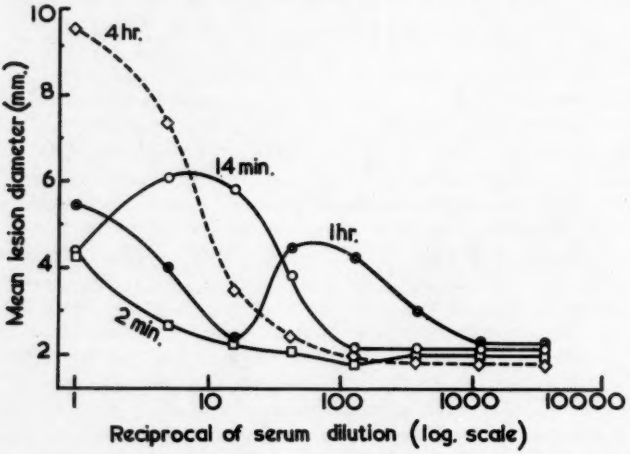

FIG. 1.—The progressive activation of PF/Dil in dilutions of fresh rat serum; dilutions in Locke's solution were tested after 2 and 14 min., 1 and 4 hr. at room temperature.

It was therefore reasonable to suppose that substances corresponding to the PF/Dil could be isolated from rat serum, and that an IPF analogous to guinea-pig IPF would be obtained by applying the methods of fractionation successful with guinea-pig serum.

Rat PF activated during serum fractionation

Rat blood was collected by cutting the throats of stunned rats and the serum harvested after holding the clotted blood overnight at 4°. In all, 5 pools of 120–270 ml. were fractionated as described by Mackay (1955) and by Wilhelm and his colleagues (1957).

The three main fractions of rat serum were similar in composition to those from guinea-pig serum: G2, mainly α- and β-globulins, in the proportion of about 2 : 1; G3, mainly γ-globulin; and AP, mainly albumin (Tables II and III). In two batches, G2 had 10 times the PF potency of G3, and AP was inactive in concentrations up to 1·5 mg./ml. Further fractionation of G2 yielded G2/1 and G2/2 (Wilhelm *et al.*, 1957) in which the ratios of α- and β-globulins were similar

TABLE II.—*Electrophoretic Protein Content of Various Rat Sera and Serum Fractions*

Batch No.	Material	Percentage protein			
			Globulins		
		Albumins	α	β	γ
3	Serum	63	12·6	18	6·4
	G2	—	63	37	—
	G3	6·5	—	—	93·5
	AP	91	9	—	—
8	Serum	63	21	10	8
	G2/1	4	64*	24	11
	G2/2	—	65	36	—
	G3	—	2	1·5	95
	AP	89	6	5	—
9	Serum	72†	6·5	14	8
	G2/1R	—	69	31	—
	G2/2	—	71	29·5	—
	G3	—	9	15	76
	AP	88·5	8	3·5	—

* The 64 per cent of α-globulin consisted of 9 per cent α_1-globulin and 55 per cent α_2-globulin.
† The figure includes some α_1-globulin, which was not fully resolved in this serum.

TABLE III.—*Alpha-Globulin Content of Fractions of Rat Serum (Batch No. 8)*

Fraction	Nitrogen (mg.)		Percentage of total α-globulin
	Total	α-globulin	
Serum	7400	1550	100
G2/1	985	630	40
G2/2	660	428	29
G1S/P	124	nt	nt
G3	325	6	—
AP	4250	255	16

nt = not tested.

to that in G2; but G2/2 had less than one per cent of the PF potency of G2/1. The reprecipitation of G2/1 yielded the fraction G2/1R, which had about twice the PF potency of G2/1 (Table IV). G2/1R, of which the average EBD was consistently about 3 μg., was adopted as the routine preparation of rat serum PF. By electrophoretic fractionation in the preparative Tiselius apparatus, G2/1R (Batch 9) was divided into preparations consisting predominantly of α- and β-globulins. G2/1Rα had 12 times the potency of G2/1Rβ (Table IV). Relative to histamine, G2/1Rα had a PF potency of 0·44, a figure remarkably similar to that for guinea-pig PF tested in the guinea-pig (Wilhelm *et al.*, 1955, 1957). Electrophoretic fractionation of a batch of G2/1R by the starch-block method (Kunkel and Slater, 1952) yielded an α- and a β-globulin fraction; the α-fraction was 47 times more potent than the β-fraction, and 40 times more potent than an eluate obtained from the block in the zone between the α- and β-bands.

Our best rat preparation, one of PF/G2α, had 44 per cent of the activity of histamine in rat skin and contained 620 EBD/mg.

Serum content of activable PF.—In the most successful fractionation of serum (Batch 8) containing 62 mg./ml. protein, 85 per cent of the serum proteins were

TABLE IV.—*The Percentage PF Potency of Various G2 Fractions and of Histamine in the Rat, Relative to a Standard Preparation of Guinea-pig PF/G2*

Batch No.	Material	Relative PF potency
Rat 2	G2	8·5
„ 3	G2	12
„ 7	G2/1	12
„ 8	G2/1	16
	G2/2	0·1
„ 9	G2/1R	35
	G2/2	0·3
	G2/1Rα	56
	G2/1Rβ	4·7
Guinea-pig—		
33 (standard)	G2/1R	100
	Histamine	130

recovered; 98 per cent of the PF activity was in the G2/1 fraction, which contained (Table II) 13·3 per cent of the original serum protein. The EBD of the G2/1 was 3 μg., so that 1 ml. serum yielded about $62 \times 0{\cdot}133 \times 10^3/3 = 2750$ EBD of PF. It appears that at most, rat serum contains some 3000 EBD of activable PF per ml.

This is a much larger figure than the 500 EBD PF/Dil activable by dilution of 1 ml. serum. Nevertheless, the similarity of properties of PF/Dil and PF/G2 (see below) strongly suggest that the two are identical. The difference is probably a consequence of the observed transience of PF/Dil in serum dilutions either because of an inherent instability, or a high susceptiblity to native PF inhibitor.

Properties of rat PF/G2

The properties of rat PF/G2 described below are in fact those of the fractions G2/1 and G2/1R; but it is convenient to use the simpler designation PF/G2 already established for the guinea-pig.

Since the PF fractions are prepared by precipitation with an organic solvent under conditions which precipitate lipo-proteins but leave smaller molecules in solution; and since in the final stage of preparation, G2 fractions are dialysed for 3 days against 6 changes of buffer, it appears that PF/G2 is a large-molecular substance. The results of electrophoretic fractionation indicate that it is either an α-globulin or intimately associated with the α-globulins.

Stability to storage.—Rat PF/G2, like guinea-pig PF/G2, is stable at $-10°$ in 2–4 per cent solution for at least 2 years.

Stability to heat.—Over the range of concentrations 18–486 μg./ml. there was no loss of PF activity after 30 min. at 65°; but a loss of 60 per cent after 10 min. at 80°, of 70 per cent after 2 min. at 98–100°, and of 80 per cent after 5 min. at 98–100°. Similar results were obtained by assay in the guinea-pig.

Stability to pH.—Tested in the guinea-pig, rat PF/G2 in a concentration of 1·5 mg./ml., after exposure to pH 4, 5 and 9 for 3 days at 4° with subsequent dialysis against phosphate buffer pH 8, I = 0·2, was stable at pH 5 and 9, but lost 70 per cent of its potency at pH 4.

Dosage-response to intracutaneous PF/G2.—PF/G2 rapidly induces a round area of intense blueing within 2–3 min. of injection. As noted above, the lesion-diameter is linear with respect to log. dose within the range 4·0–9·0 mm.; when

the lesion-diameter is greater than 9 mm., the response-curve flattens (Fig. 3), though not as suddenly as in the corresponding curve for guinea-pig PF/G2 in guinea-pig skin. The break in the linear response to guinea-pig PF at the 9 mm. level was attributed to the high affinity of the PF for the skin tissue, which restricted its spread beyond the area of the 9–10 mm. bleb produced at the time of the injection (Wilhelm *et al.*, 1955). The less sharp break at the same level in the response-curves to rat PF is consistent with this view, because rat PF in a constant dose titration (Miles and Miles, 1952) proved to have a lower affinity than guinea-pig PF for homologous skin tissue.

Time-course of intracutaneous PF/G2.—The increased capillary permeability induced by PF/G2, in concentrations up to 324 μg./ml., was maximum when the injections were 0–3 min. old before the rats were blued, and declined to normal low permeability within 15 min. In this respect rat PF/G2 resembles histamine and guinea-pig PF/G2 (Wilhelm *et al.*, 1955). The increased permeability induced by a high concentration, 3 mg./ml., lasted 25–30 min.

Effect on diapedesis of leucocytes.—Intracutaneous injections of graded doses in 0·1 ml. saline of rat PF/G2 were made into 9 rats and the injection sites excised for histological examination after 60 min. No substantial tissue leucocytosis was induced by rat PF/G2, 0·25–10 mg./ml.; by guinea-pig PF/G2, 0·5 mg./ml.; by histamine, 0·5 mg./ml.; or by physiological saline. Slight to moderate tissue leucocytosis was present in skin sites injected with leukotaxine, 0·3–8 mg./ml.

Susceptibility to various inhibitors.—Guinea-pig PF is characterized by high susceptibility to SBTI, slight susceptibility to PTI and insusceptibility to ovomucoid and mepyramine. The IPF in high concentrations of the fraction G1S/P (0·165 per cent) acts relatively slowly on guinea-pig PF *in vitro* at room temp., diminishing the activity by 90 per cent in 45 min. (Wilhelm *et al.*, 1955).

The pattern of inhibition of rat PF/G2 by these substances, tested in both rats and guinea-pigs, was largely similar. Mixed with a few multiples of the EBD, SBTI inhibited about its own weight of PF/G2. PTI in concentrations up to 6 μg./ml. only slightly inhibited the same dose of PF/G2 ; 18 μg./ml. inhibited 50 per cent of the dose ; PTI in concentrations greater than 18 μg./ml. could not be tested because it had considerable PF potency. Rat PF/G2 in a dose of about 3 EBD was as susceptible to guinea-pig IPF as an equipotent dose of guinea-pig PF/G2 (see Fig. 4A and p. 240). Ovomucoid is a relatively specific inhibitor of trypsin (Fraenkel-Conrat, Bean and Lineweaver, 1949). Ovomucoid itself increased permeability in concentrations of 162 μg./ml. In maximum non-blueing concentration, it inhibited 9 EBD of a preparation of trypsin and failed to affect 3 EBD of rat PF/G2, tested in guinea-pig skin.

Effect of the antihistaminic, mepyramine maleate

Mepyramine given intraperitoneally in the high doses known to affect histamine-liberators like compound 48/80 is rather toxic to the rat (Sparrow and Wilhelm, 1957). By injecting mepyramine mixed with the PF under test, it was possible in the guinea-pig to use local concentrations up to 162 μg./ml. of the antihistaminic (Wilhelm *et al.*, 1957) ; in higher concentrations local mepyramine was itself a permeability factor. Similar tests proved to be possible in the rat ; 18 μg./ml. mepyramine decreased the potency of 5 EBD of rat PF/G2 by 50 per cent ; but the potency remained at this level when the concentration of mepyramine was increased

to 486 μg./ml., suggesting that the PF effect of the G2 fraction was due to 2 substances, the less potent being a histamine-liberator.

In PF inhibition tests with local inhibitors, the periphery of the lesion blues first, and the initially uncoloured central area blues fully within 15–20 min. Inhibition is estimated solely in terms of the decrease of lesion-diameter determined after 30 min. With mixtures of mepyramine and PF/G2 in the rat, the central unblued area is persistent. We have no evidence to decide whether this effect is due to a true inhibition of a PF effect, or an interference with blood supply (*e.g.*, by vasoconstriction) by a high central concentration of mepyramine. Its occurrence does not affect our measure of inhibition in terms of a decrease in overall diameter, but it may well be additional evidence that PF/G2, at least in part, increases permeability by the liberation of histamine.

Rat PF/G2 as a histamine-liberator.—The liberation of histamine by rat and guinea-pig PF/G2 was investigated as follows. Histamine was assayed on preparations of atropinized guinea-pig ileum.

(i) Perfusion of rat and guinea-pig hind quarters preparations (Feldberg and Mongar, 1954). Five rat hind quarters preparations were each perfused with 1 mg. rat PF/G2 (≡ 250 rat EBD), and six with 1 mg. guinea-pig PF/G2 (≡ 1100 rat EBD). No histamine was detected in the perfusates, but in each case subsequent perfusion with 2 μg. compound 48/80 (≡ 13 rat EBD) liberated histamine as abundantly as from hitherto untreated preparations. Similarly 2 mg. rat PF/G2 (≡ 3300 guinea-pig EBD) failed to liberate histamine from a guinea-pig preparation, and 1–2 mg. guinea-pig PF/G2 (≡ 25,000–50,000 guinea-pig EBD) failed to do so from three other guinea-pig preparations.

(ii) Incubation with rat skin, uterus and lung and with guinea-pig skin (Schild, 1939). The tissue preparations were held for 30 min. at 37° in 10 ml. Locke's solution. Neither 4 mg. rat PF/G2 (≡ 1000 rat EBD) nor 2 mg. guinea-pig PF/G2 (≡ 2200 rat EBD) liberated histamine from rat skin, lung or uterus, in conditions where substantial amounts were subsequently liberated by 20 μg. 48/80 (≡ 130 rat EBD). Similarly 4 mg. guinea-pig PF/G2 (≡ 100,000 guinea-pig EBD) failed to liberate histamine from guinea-pig skin.

(iii) Injection into the peritoneal cavity of the rat (Fawcett, 1954). The injection of 200 μg. 48/80 (≡ 1300 rat EBD) in 13·4 ml. Locke's solution into 200 g. rats of our laboratory strain within 30 min. disrupted almost all the subendothelial mast cells in the mesentery and liberated on the average 5 μg. histamine into the injection fluid. In a similar injection-volume, 13–26 mg. of rat PF/G2 (≡ 1100–2200 rat EBD) did not affect the mast cells or liberate any detectable histamine.

It is clear from the EBD equivalents of the doses employed that the amounts of G2 fractions tested had a total PF activity that was either substantially greater than sections (i) and (ii) or at least equal to section (iii) the PF activity of the 48/80 used as a control histamine-liberator. It is therefore unlikely that the G2 preparations owed their PF activity to a frank release of histamine in the way that 48/80 does. The results of directly measuring histamine release from susceptible tissues cast serious doubt on the specificity of the relatively small *in vivo* inhibition of rat PF/G2 by mepyramine. On the other hand, our failure to detect liberation may have been due to the choice of an unsuitable test system, because, as Feldberg and Mongar (1954) record, established histamine-liberators like 48/80 and octylamine can vary 1000-fold in their relative capacities to liberate histamine, according to the tissue preparation used in the test.

(iv) Reactions in the skin of histamine-depleted rats. Another line of investigation was indicated by the observation of Brocklehurst, Humphrey and Perry (1955) that depletion of the skin histamine of rats by the continued administration of compound 48/80 caused a decrease in the potency of 48/80 as a permeability factor in the skin of blued animals. Accord-

ing to these results, any element of histamine liberation in the action of PF/G2 should be evident from a lowering of its potency in depleted rats.

Twenty-five rats were given a 10-day course of intraperitoneal 48/80 (Sparrow and Wilhelm, 1957); on the 1st, 2nd, 3rd, 5th and 8th days after cessation of the treatment, groups of 4 rats were blued and tested intracutaneously with histamine, 48/80, 5-hydroxytryptamine (5-HT) and rat and guinea-pig PF/G2. A control group that had received a parallel course of intraperitoneal saline equal in volume to the solution of 48/80 given to the treated animals was tested on the 2nd day; and the PF activities in the treated group expressed as a percentage of the activities in this control group (Fig. 2).

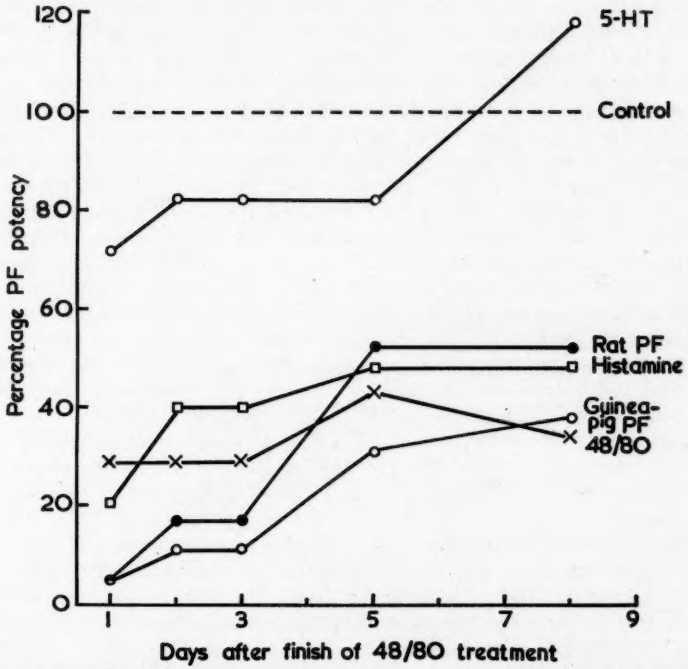

FIG. 2.—The depression and subsequent recovery of the response of rat skin to various permeability factors, 1–8 days after a ten-day course of compound 48/80 intraperitoneally.

The potencies of 48/80, and 5-HT, both acknowledged histamine-liberators, and of the rat and guinea-pig serum PF were all decreased on the 1st day; and, as Brocklehurst and his colleagues (1955) found, so was the potency of histamine. This last non-specific effect may be a reflection of the depressed state of the rats owing to the cumulative effect of treatment with 48/80. We obtained similar results on the 1st day in 2 other experiments (Sparrow and Wilhelm, 1957), including a 95 per cent depression of the response to guinea-pig PF/G2, a substance that so far has proved to be insusceptible to mepyramine.

According to Brocklehurst, Humphrey and Perry, the rats recover their normal response to histamine on the 2nd day. At this time the response to histamine in our rats (Fig. 2) was still less than 40 per cent of its usual value, and even on the 8th day was only 50 per cent. The response to the two serum PF improved to about the same degree. On the other hand, that to 48/80 remained relatively steady at 30 per cent of its control value, a fact consistent with the observation of Feldberg and Talesnik (1953) that the skin of the rat depleted of 90

per cent of its histamine does not begin to acquire new histamine until 10 days or more after the cessation of the systemic treatment with 48/80.

Our reliance on a single test of control animals on the 2nd day was justified by the steady performance of our laboratory strain of rats in other assays for several months immediately before and after the experiment. The deviation from the normal in the response *e.g.*, to histamine in the treated animals, even on the 8th day, was strikingly greater than that to be expected from the small secular variability of the normal response that we observed during the rest of this period.

In summary, PF/G2, from either rat or guinea-pig fails to liberate demonstrable histamine from rat or guinea-pig tissues in biological systems highly susceptible to the action of the histamine-liberator, 48/80. This result is difficult to reconcile with the action of mepyramine on rat serum permeability factors. If rat PF/G2 is not a histamine-liberator, its susceptiblity to local mepyramine is perhaps moderate enough to be explained as non-specific. But the inhibition of rat PF/Dil by mepyramine is too great to be lightly dismissed in this way. The inhibition may indeed prove to be non-specific in both cases, but if it is specific with PF/Dil, it is likely to be so with PF/G2, since by their similarity in other tests we have applied and by analogy with the PF/Dil and PF/G2 of guinea-pig serum, rat PF/Dil and rat PF/G2 appear to be identical. In this case, serum dilutions with PF/Dil activity and preparations of G2 may both contain 2 permeability factors only one of which is a histamine-liberator ; or rat PF, whether in PF/Dil or PF/G2, is a single substance acting in two ways, one of which entails histamine liberation. The evidence at present is insufficient to decide between these alternatives. As regards the depression of the PF/G2 response in histamine-depleted rats, it appears in our hands to be non-specific and is therefore not acceptable evidence of the dependence of the G2 permeability effect on histamine release.

Inhibitor of 5-hydroxytryptamine (*5-HT*).—In view of the demonstration by Rowley and Benditt (1956) that 5-HT is a potent permeability factor in the rat, we tested the effect on PF/G2 of the 5-HT inhibitor, lysergic acid diethylamide (LSD). Graded concentrations of LSD, 6–162 μg./ml. were held with PF/G2 for 30 min. at room temperature and injected intracutaneously with due precautions against systemic effects resulting from the absorption of LSD from the higher local concentrations (Sparrow and Wilhelm, 1957). The results were clear cut ; LSD, in 10 times the concentration required to inhibit equipotent PF doses of 5-HT, had no effect on rat PF/G2.

Relation to the plasmin system.—The inhibition of rat PF/G2 by SBTI suggests that, like guinea-pig PF/G2, it is a protease and therefore may be plasmin, which is known to be present in G2 fractions.

Plasmin, like PF, is inhibited by SBTI, but the two activities change disproportionately when G2 preparations are heated. After 10 min. at 80° a preparation of G2/1R, 1·46 mg./ml., lost 60 per cent of its PF activity and 98 per cent of its caseinolytic activity ; after 30 min. at 65°, the corresponding losses were 0 per cent and 90 per cent (Fig. 3). The retained PF activity of the heated preparations presumably was not due to breakdown products, because like the unheated controls, they were susceptible to SBTI. Thus, like guinea-pig PF, rat PF does not appear to be plasmin.

Hypotensive effect.—Rat G2 preparations are hypotensive on intravenous injection into rats and guinea-pigs. In our study of guinea-pig PF (Wilhelm *et al.*, 1955) the G2 fraction induced an acute hypotension on intravenous injection in the guinea-pig, dog and rabbit ; and from the apparent parallelism of PF and

hypotensive potency in a limited number of G2 preparations, we concluded that the PF and the hypotensive factor (HF) were identical. A similar study (to be published) of rat G2, and further study of the guinea-pig fraction G2 suggests that the HF is probably distinct from the PF. The hypotensive effect is not considered further in this paper.

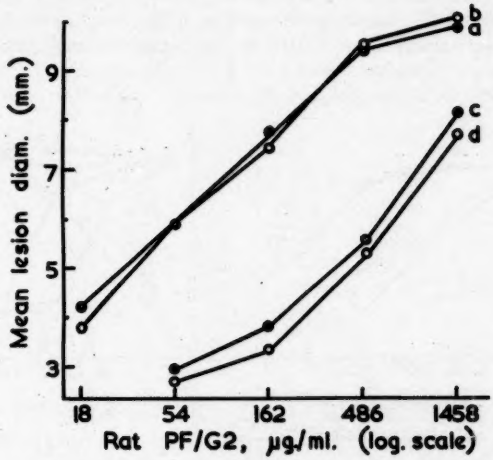

FIG. 3.—The heat-stability of rat serum PF and susceptibility to soya bean trypsin inhibitor (SBTI).
a = untreated fraction PF/G2.
b = PF/G2 heated 30 min. at 65°.
c = unheated PF/G2 + SBTI, 18 µg./ml.
d = heated PF/G2 + SBTI, 18 µg./ml.

Isolation and properties of rat IPF

G1S/P, the fraction devised for the isolation of IPF in guinea-pig serum (Wilhelm *et al.*, 1955) proved to have the greatest IPF potency of all the fractions of rat serum, although some was present in the crude albumin fraction AP. The AP fraction had one-third the potency of G1S/P; the remaining fractions G2 and G3 themselves had PF activity and could not be tested for IPF potency.

Electrophoretically, G1S/P and AP were alike in containing 6 per cent α_2-globulin and 3·5 per cent β-globulin. They differed in that G1S/P contained 85 per cent (α_1-globulin + albumin) and 5·5 per cent γ-globulin, whereas AP contained 90 per cent (α_1-globulin + albumin) and no γ-globulin. In both, the content of α_1-globulin was of the order of 1–2 per cent. Further analysis of the G1S/P fraction by starch block electrophoresis indicated that the IPF was present in both the α_1-globulin and the albumin regions. We have as yet no evidence to decide whether the IPF is associated exclusively with either the α_1-globulins or the albumins.

Like guinea-pig IPF, rat IPF acts relatively slowly *in vitro*. For example, six separate samples of rat PF/G2, in a final concentration of 250 µg./ml., were mixed at hourly intervals with equal volumes of rat G1S/P in a final concentration of 4·3 mg./ml.; all mixtures were held at room temperature and tested in blued

rats at the 6th hr. Little inhibition was evident before the lapse of 1 hr. at room temperature. The percentage inhibition was 58 after 1–2 hr., 78 after 3–4 hr. and 85 after 6 hr.

Relation to guinea-pig IPF.—The relationship of rat and guinea-pig IPF and PF are displayed in Fig. 4A and 4B, which record the inhibition, after 45 min. *in vitro* at room temperature, of equipotent doses of rat and guinea-pig PF by rat and guinea-pig IPF, as determined in both test animals. Several points emerge from these experiments. In the first place, preparations of guinea-pig

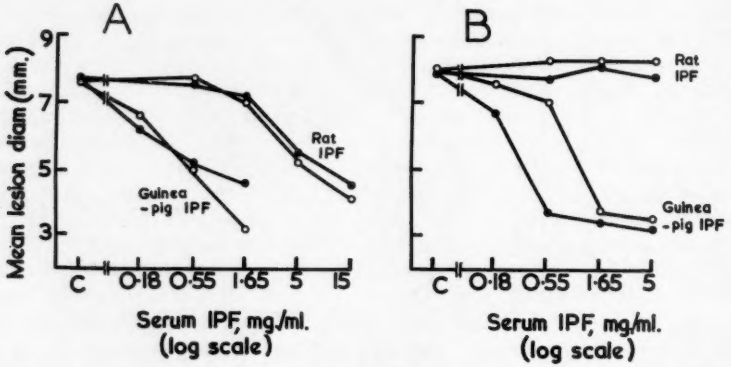

FIG. 4.—The susceptibility of rat PF (●——●) and guinea-pig PF (○——○) to rat and guinea-pig IPF when tested in rat skin (A) and their susceptibility only to guinea-pig IPF when tested in guinea-pig skin (B). C = control.

IPF are always more potent than those of rat IPF; tested in rats the difference is 14-fold (Fig. 4A), and in guinea-pigs more than 28-fold (Fig. 4B). In the second, both permeability factors are equally susceptible to a given dose of either rat or guinea-pig IPF, but only when the test is made in rat skin. Tested in guinea-pig skin, rat IPF has no action on either rat or guinea-pig PF; and, whereas guinea-pig IPF affects them both, it is 3 times more effective on rat PF than on guinea-pig PF. Thus, the potency of the two inhibitors varies, not only with the species of PF, but also with the animal whose skin is used to measure the residual PF activity of the PF/IPF mixtures. Nevertheless, it appears that the action of rat IPF on rat PF, tested in the rat, is closely analogous with that of guinea-pig IPF on guinea-pig PF, tested in the guinea-pig.

Rabbit Serum

A native PF (PF/Nat) and the absence of a PF activable by dilution

PF/Nat.—Fresh undiluted rabbit serum, harvested from a clot or by centrifugation of defibrinated blood, contains a strong native PF (PF/Nat) whose activity disappears on dilution of the serum in saline to 1/45 (Fig. 5). Rabbit PF/Nat is also active in the guinea-pig and rat. This PF may be in part analogous to the PF/Nat of undiluted rat serum, and to the PF/Age of undiluted guinea-pig serum. Like them, it is insusceptible to guinea-pig IPF and to SBTI, and there-

fore does not correspond with the main PF in rat and guinea-pig sera ; but in some of the rabbit sera tested it differed from PF/Age and PF/Nat of the other species in being susceptible to local mepyramine, 162 μg./ml. Thus in two fresh sera, PF/Nat was inhibited entirely by treatment with mepyramine. Two other sera were found to contain respectively the equivalent of 1·3 and 2·7 μg./ml. histamine, assayed on preparations of atropinized guinea-pig ileum. In yet other fresh sera, harvested by the same technique from animals in the same state of health, only a little of the PF/Nat activity was inhibited by mepyramine, the remaining PF thus resembling the PF/Age of guinea-pig serum and the PF/Nat of rat serum. Rabbit blood is reported (Erspamer, 1954) to contain about 3·5

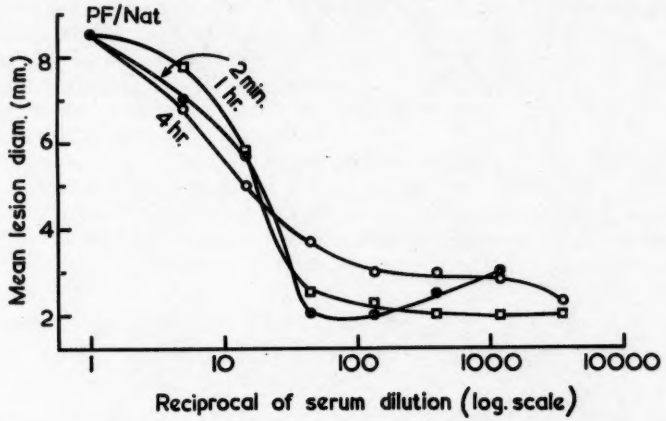

FIG 5.—The absence of a dilution effect on the permeability factors of rabbit serum. Dilutions in saline were tested after 2 min., 1 hr. and 4 hr. at room temperature.

μg. 5-HT/ml. The PF not affected by mepyramine in rabbit serum is not likely to be 5-HT since in concentrations up to 500 μg./ml. 5-HT has no PF potency in the skin of the rabbit (Sparrow and Wilhelm, 1957).

One EBD of PF/Nat occurred in dilutions of 1/15–1/45, even when the lesions were allowed to mature for 4 hr. (see p. 240) ; *i.e.*, 1 ml. serum contains some 450 EBD PF/Nat. Since this represents about 3 per cent of the total (p. 246) PF substance isolable from rabbit serum, PF/Nat was not further investigated.

PF/Dil.—All attempts to activate a PF/Dil, using the rabbit as the test animal, were failures. Dilution series of fresh rabbit serum, aged 2 min. to 3 days exhibited only PF/Nat, even when the lesions were read after 4 hr. Similar tests of the dilution series in the guinea-pig were negative, except on one occasion when a PF/Dil peak appeared at 1/405 in dilutions 12 min. old.

The activation of PF/Dil in guinea-pig serum is enhanced when dilutions are dialysed against saline for 3 days at 4° (Wilhelm *et al.*, 1957). Dilutions of previously dialysed rabbit serum, and dialysed dilutions of untreated serum, were tested in the guinea-pig with negative results except on one occasion, when a peak of activity appeared in the range 1/45–1/405. It is clear that if the main rabbit PF is indeed activable by dilution, we have not yet mastered the conditions of activation.

Rabbit PF activated during serum fractionation

The results of fractionating a number of pools of fresh rabbit serum by the method of Mackay (1955) and in some cases by the modification of this method finally adopted for guinea-pig serum (Wilhelm *et al.*, 1957) are set out in Tables V and VI.

TABLE V.—*Electrophoretic Protein Content of Various Rabbit Sera and Serum Fractions*

Batch No.	Material	Percentage protein			
			Globulins		
		Albumins	α	β	γ
3	Serum	68	6	13	13
	G2	—	55	45	—
	G3	4	—	—	96
	AP	90	10	—	—
9	Serum	68	8	10	12
	G2/1	—	52	41	8
	G2/2	2	57	39	—
	G3	7	12		83
	AP	84	10	4	—
10	Serum	68	7	13	10
	G2/1R	—	54	33	12
	G2/2	—	59	41	—
	G3	—	4	13	83
	AP	90	3	7	—

TABLE VI.—*The Percentage Potency of Various G2 Fractions and of Histamine in the Rabbit, Relative to a Standard Preparation of Guinea-pig PF/G2*

Batch	Material	Relative PF potency*
Rabbit 3	G2	5·5
„ 5	G2	7·6
„ 8	G2/1	14
	G2/2	22
„ 9	G2/1	15
	G2/2	15
„ 10	G2/1R	74
	G2/2	21
Guinea-pig—		
33 (standard)	G2/1R	100
	Histamine	29,000

* Thirty minute readings.

In three batches, G2 fractions had 5–10 times the PF potency of G3 and AP; but the results of fractionating G2 itself were not always consistent. Sometimes G2/1 and G2/2 had the same PF activity. Only in the fractionation of Batches 10 and 11, in which the starting material was serum not more than 2 hr. old, did we succeed in concentrating the PF in G2/1, the potency ratio of G2/1 to G2/2 being 3·6 : 1 and 12 : 1 respectively. In guinea-pig and rat serum fractions, potency ratios of this order and higher were consistently obtained. The potency of rabbit G2/1 was improved 5-fold by reprecipitation as the fraction G2/1R.

Properties of rabbit PF/G2

The properties of rabbit PF/G2 were determined with our most potent preparations, the fractions G2/1 and G2/1R. As with the rat PF/G2, the method of preparing fractions that contain rabbit PF suggests that it is a large-molecular substance, but its electrophoretic definition is not clear-cut. Electrophoretic fractionation at pH 8·0 of G2/1R of Batch 10 in the preparative Tiselius apparatus yielded a predominantly β-globulin fraction with a potency of 170 and an α-globulin fraction with a potency of 44 (guinea-pig PF standard = 100). This association of the PF with β-globulin could not be confirmed by electrophoretic separation of the same batch of G2/1R on a starch block at the same pH; the α-globulins had 4 times the potency of the β. When G2/1R of Batch 11 was separated on a starch block at pH 8·0 and 9·1, there was no substantial difference between the potencies of proteins in the α-, $\alpha\beta$- and β-globulin bands, although at pH 9·1 (in glycine buffer) the α- and β-globulins were widely separated. It appears, therefore, that the rabbit serum PF, if it is indeed one molecular species, has the mobility of both α- and β-globulins.

Duration of effect.—The G2 fractions were initially assayed in the rabbit by a method similar to that used for guinea-pig and rat, including a routine reading of the blue lesions after 30 min., at which time the lesions in the guinea-pig and rat are fully developed. By this method the relative potencies of the various fractions listed in Table VI were obtained. However, the duration of increased permeability

TABLE VII.—*The IPF Potency of Various Fractions of Rabbit Serum, Measured in the Rabbit Against a Constant Amount of Rabbit Serum PF, and Estimated from Readings made at ½, 2 and 4 hr.*

Inhibitor tested			Percentage inhibition of PF estimated from readings at (hr.)		
Batch No.	Fraction	Conc. (mg./ml.)	½	2	4
10	G1S/P	0·18	44	68	62
		1·65	58	47	53
	AP	0·18	40	68	53
		1·65	44	40	0
11	G1S/P	0·18	44	60	40
		1·65	66	40	40
	AP	0·55	33	44	33
		1·65	40	61	55
		5·00	44	55	37
12	I	1·08	92	65	60
	II	1·08	85	25	20

induced by rabbit PF/G2 proved to be maximum at 4 hr., in contrast to the 2–3 min. for guinea-pig PF/G2 in the guinea-pig (Wilhelm *et al.*, 1955). Fig. 6 records a test in which dye was given intravenously to rabbits already bearing skin lesions of graded ages, and the readings were made 30 min. later. The long-lasting action is a peculiarity of rabbit serum PF, because other permeability factors have much shorter time-courses. By contrast, histamine in rabbit skin has the

characteristic short time-course that it has in the guinea-pig ; that of guinea-pig PF/G2 is longer in the rabbit than it is in the guinea-pig, but even so, only the faintest residual blueing occurs in lesions more than 1 hr. old. The rabbit PF/G2 lesions, on the other hand, increase in size for over 4 hr. Although the lesion size is maximum at about the 4th hr., the demonstrable duration of increased permeability in a given region of the injection site is only 2–2½ hr. As the interrupted curve in Fig. 6 indicates, no dye enters the tissues at the centre of the lesion after 2 hr. This central unblued area grows larger, so that at the 4th hr. only a thin annulus of blued skin appears on injection of the intravenous eye. At the

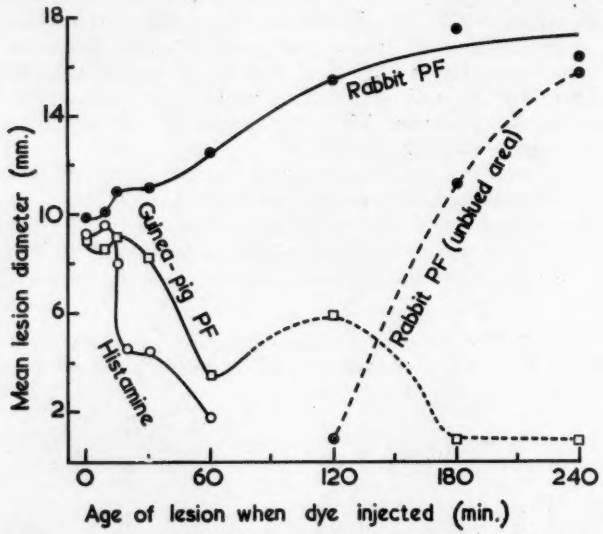

Fig 6.—The prolonged time-course in the rabbit of rabbit serum PF, compared with that of guinea-pig serum PF and of histamine. The intravenous dye was given to animals already bearing lesions of the indicated ages. After 2 hr., an area which does not blue develops in the centre of the rabbit PF lesions.

3rd hr. the tissues 5·5 mm. from the centre of the lesion (11 mm. lesion-diameter) cease to blue ; but they only begin to do so 2½ hr. earlier, at 30 min. Similarly the tissues 7·5 mm. from the centre (15 mm. lesion-diameter), which fail to blue at the 4th hr., do not start to do so until the 2nd hr.

A striking consequence of the relatively prolonged reaction to rabbit PF is displayed in Fig. 7, which records the gradual increase in size of the blue lesions in animals with dye in the circulation at the time of the intracutaneous injections. Doses which do not visibly increase permeability within 30 min. induce lesions 13 mm. in diameter in 4 hr. The increase in lesion size during the 4 hr. reflects a steady expansion of the area of increased permeability. The alternative explanation, of a peripheral spread from the centre of the lesion of an excess of already-exuded dye, is excluded by experiments like that recorded in Fig. 6, where the large 4 hr. lesions induced by rabbit serum PF are rapidly blued at the periphery by dye introduced into the circulation only 30 min. previously.

The details and implications of species differences of this kind in skin reactions to serum PF will be discussed more fully in a subsequent paper. At this point, it is sufficient to emphasise that, in the guinea-pig and rat, any increase of lesion size after 30 min. appears to be due to the outward diffusion of blue exudate from the original area of capillary change, whereas in the rabbit the duration of the permeability effect indicates that it is due to a response to the PF in areas of the vascular bed that hitherto had not responded. The 30 min. reading, therefore,

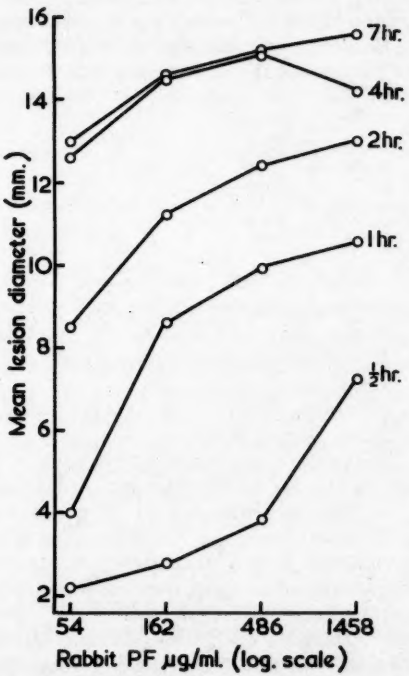

FIG. 7.—The maturation during a 7 hr. period of the increased permeability induced by graded doses of rabbit serum PF in rabbits with dye already in the circulation.

is a valid basis for potency assays in the guinea-pig and rat—including assays of rabbit PF in these species—but not in the rabbit.

In rabbits yielding results like those in Fig. 7, the potency of PF/G2 estimated from ½ hr. readings is as little as 1/200th of the true potency. In other animals, particularly those tested during the moulting season, the "½ hr." potency is often as much as a tenth to a quarter of the true potency. But at all times of the year the responses of the rabbits to homologous serum PF and the rate of development of the maximum permeability are liable to variation. The effect of the large secular variability we observed was minimized by adopting the 4 hr. reading, and was countered as far as possible by the routine use of both histamine and guinea-pig PF/G2 as standard preparations.

Dosage-response to intracutaneous PF/G2.—As with guinea-pig and rat PF/G2 tested in the homologous animals, within the range 4·0–8·5 mm. the lesion-diameters induced by rabbit PF/G2 are linear with respect to log. dose and the response-lines have slopes of 3·5–5·0. The same relations hold for 4 hr. readings, though, as Fig. 7 shows, the doses which at 4 hr. induce responses within the useful range 4·0–8·5 mm. are inactive at 30 min.

Serum content of activable PF/G2.—In the most successful fractionation of serum Batch 10, containing 72 mg./ml. protein, 56 per cent of the serum proteins was recovered ; 80 per cent of the PF activity was in the fraction G2/1R, and the remaining 20 per cent activity was distributed among the three other fractions as follows (the EBD were estimated from the results of 30 min. readings).

Fraction	Weight recovered (mg.)	EBD (μg.)	Total EBD
G2/1R	3·19	0·25	12,760
G2/2	3·12	1·60	1,950
G3	3·54	4·0	810
G1S/P	2·98	6·3	480

Summing the last column, we find that one ml. serum yields 16,000 EBD. Allowing for the fact that the greater part of the 44 per cent loss during fractionation was probably of albumin, and that PF activity is associated with the α- and β-globulins, it appears that rabbit serum contains 20,000–25,000 EBD of activable PF per ml.

Stability to storage.—Rabbit PF/G2 in 2–3 per cent solution retains its PF potency at −10° for at least 18 months.

Stability to heat.—Estimated from 30 min. readings of the lesions, the heat stability resembles that of the rat and guinea-pig PF. There was no loss of PF activity after 30 min. at 65° ; but a loss of 20–30 per cent after 10 min. at 80°, of 70 per cent after 2 min. at 98–100°, and of 100 per cent after 10 min. at 98–100°. When the more sensitive assay with 4 hr. readings was performed, the degree of inactivation at the lower temperatures was correspondingly less ; but exposure for 10 min. at 98–100° destroyed the " 4 hr." as well as the " 30 min." PF activity.

Tested in guinea-pigs, rabbit PF/G2 was not affected by heating at 65° for 30 min. or at 80° for 10 min., but lost 45 per cent of potency at 98–100° for 2 min.

Stability to pH.—Rabbit PF/G2, 1·5 mg./ml. was exposed to pH 4, 5 and 9 for 3 days at 4° with subsequent dialysis against phosphate buffer pH 8, I = 0·2. Tested in guinea-pigs, PF/G2 was stable at pH 9, but lost 25 per cent of its potency at pH 5 and 45 per cent at pH 4.

Susceptibility to various inhibitors.—The PF potency of guinea-pig and rat PF is decreased 90 per cent or more by 18–54 μg./ml. SBTI and by 0·55–1·65 mg./ml. guinea-pig IPF ; and rat PF, but not guinea-pig PF, is moderately susceptible to mepyramine. In contrast, rabbit PF is moderately susceptible to large doses of all three inhibitors. The inhibition is of the same order when the residual PF activity is estimated from 30 min. or 4 hr. readings. It ranges from 0–40 per cent using 162 μg./ml. SBTI, and from 40–60 per cent using 5 mg./ml. guinea-pig IPF and 1458 μg./ml. mepyramine. Inhibition of the order of 50 per cent with an average slope of the dosage-response line of 3·5–4·0 represents a decrease in mean lesion-diameter of about 1 mm.; and although we have established the reality of the decrease by repeated tests, it is of the order of size encoun-

tered in non-specific depression of the animals' capacity to react to permeability factors. That it represents a true inhibition is evident from the greater efficacy of the inhibitors when tested with rabbit PF in the skin of guinea-pigs and rats. In the guinea-pig, the inhibition of rabbit PF by SBTI and by guinea-pig IPF was over 90 per cent. and in the rat inhibition by mepyramine was 85–90 per cent.

The action of the 5-HT antagonist, LSD, was not tested because 5-HT itself has no substantial potency as a permeability factor in the rabbit (Sparrow and Wilhelm, 1957).

Relation to the plasmin system.—Although rabbit PF is susceptible to SBTI, which also inhibits plasmin, G2 fractions do not appear to owe their PF activity to plasmin itself, because the caseinolytic potency of 1·46 mg./ml. of rabbit PF/G2 was strongly decreased by heat, whereas the PF activity was less affected, as follows.

Heat applied	Percentage decrease of potency	
	PF	"Plasmin"
30 min. at 65°	30	67
10 ,, ,, 80°	60	98
2 ,, ,, 98–100°	40	>99

Hypotensive effect.—On intravenous injection the serum fractions G2/1R and G2/2 induce slight to moderate hypotension in the rabbit (to be published).

Effect on diapedesis of leucocytes.—The sites of intracutaneous injections of graded doses of rabbit PF/G2 in 0·1 ml. saline into rabbits were excised after 4 hr. for histological examination. Rabbit PF/G2, 54–2916 μg./ml. induced considerable tissue leucocytosis of the dermis and subdermis, and particularly of the deeper half of the dermis and the panniculus adiposus. The largest doses induced intense leucocytosis thoughout the whole thickness of the skin. Histamine, 0·37–10 μg./ml. induced slight, and guinea-pig PF/G2, 54–1458 μg./ml. slight to moderate tissue leucocytosis.

The demonstration of IPF activity in rabbit serum

Since rabbit serum does not display the dilution effect characterizing both guinea-pig and rat serum, we had no *prima facie* grounds for supposing that any IPF was present as such. The absence of a dilution effect might be due to the presence of a highly effective IPF, which quickly antagonizes any PF activated by dilution. However, dilutions of rabbit serum in the range 1/15–1/135, which have no PF activity, did not inhibit rabbit PF/G2 when held with it for 45 min. at room temperature before intracutaneous test in the guinea-pig.

Nevertheless, using the rabbit as the test animal, an IPF active against rabbit PF/G2 can be demonstrated in fractions of rabbit serum. Two samples of fresh serum were fractionated by the routine aqueous ethyl ether method to yield the crude albumin fraction AP and the fraction G1S/P, which in the guinea-pig and rat are richest in IPF; and one sample by starch block electrophoresis in phosphate buffer pH 8·0, I = 0·1 (Kunkel and Slater, 1952) to yield two fractions; fraction I was obtained from the more mobile half of the albumin band and fraction II from the less mobile half, *i.e.*, nearest to the α-globulins.

Graded amounts of the fractions were held with rabbit G2 preparations for 45 min. at room temperature and tested in the blued rabbit. Table VII records

the approximate percentage decrease in potency of the PF with the various fractions. The relative inaccuracy of the assays does not justify putting too much weight on the individual percentage values, but certain trends are clear. As regards the ½ hr. readings, all G1S/P and AP fractions contain IPF and the larger doses are the more inhibitory. The inhibition at 2 hr. is slightly more pronounced, particularly with the smaller doses of the inhibitory fractions; but at 4 hr. the inhibition by the larger doses of IPF tends to be less. This last effect is more pronounced with the AP than with the G1S/P fraction. It also occurs with the electrophoretically separated fraction II. Since AP and fraction II alone also exhibited PF activity after this period, the effect may with some confidence be attributed to contamination of the IPF fractions with small amounts of a PF, whose action (cf. Fig. 7) becomes manifest only in lesions aged 2–4 hr.

The active inhibition by fraction I suggests strongly that, in the rabbit, IPF is associated with albumin, rather than with α-globulins, as it is in the guinea-pig. Our results confirm the report of Paskhina (1956), of inhibition by a "pure preparation" of rabbit serum albumin; but in view of the distribution of active IPF among our various fractions, more work is required to identify with any certainty the rabbit serum proteins associated with IPF activity.

SUMMARY

Our preliminary report (Wilhelm, 1956) of an activable precursor (pro-PF) of a globulin permeability factor (PF), and of an inhibitor (IPF) of this PF, in the serum of both the rat and the rabbit is confirmed. In general, the pro-PF/IPF systems in rat and rabbit serum resemble that already described in the guinea-pig; but they differ considerably in detail. The significance of our findings, and a comparative study of the systems in guinea-pig, rat, rabbit and man will be discussed in a later paper.

Fresh undiluted *rat* serum contains little PF, but on dilution to 1/45 in 0·85 per cent saline or in Locke's solution and standing for 1 hr. at room temperature yields a PF/Dil (cf. Spector, 1956), of which some 500 EBD are activable in 1 ml. serum. Rat PF/Dil, like guinea-pig PF/Dil, is antagonized by soya bean trypsin inhibitor (SBTI), but differs from it in being antagonized by the antihistaminic, mepyramine maleate. The PF/Dil disappears after 4 hr. at room temperature. The disappearance is due, at least in part, to the action of a native IPF in the serum. A feeble "native" PF (PF/Nat) in fresh undiluted serum increases rapidly in amount within a few hours of harvesting the serum; it represents about 5 per cent of the total permeability-increasing substance isolable from rat serum. In its insusceptibility to SBTI, to serum IPF and to mepyramine, PF/Nat resembles the more slowly formed minor PF/Age of ageing guinea-pig serum.

A heat stable PF is activated during fractionation of rat serum by the aqueous ethyl ether method; it appears to be a globulin and is consistently found in the α-globulin fraction; it has about half the PF potency of histamine in the rat. Like the α-globulin PF isolable from guinea-pig serum, it is rapidly acting; its permeability effect is maximum in 3 min. and lasts 15–20 min.; and it is inhibited by SBTI and by IPF. Unlike guinea-pig serum PF, it is moderately inhibited by mepyramine. Rat serum PF appears to be identical with rat PF/Dil; some 3000 effective blueing doses (EBD) can be isolated from 1 ml. serum.

Despite its moderate susceptibility to mepyramine, rat serum PF did not

liberate histamine from three kinds of test preparations of rat and guinea-pig tissues. Rat serum PF is not a liberator of 5-hydroxytryptamine ; and it is not plasmin.

The IPF active in native serum (Spector, 1956) is contained in the crude α_1-globulin and the albumin fractions. Like guinea-pig serum IPF, it has a moderate potency, and acts slowly on serum PF.

Fresh undiluted *rabbit* serum contains moderately potent native PF (PF/Nat), and sometimes histamine as well. The PF/Nat is diluted out at 1/50 and represents about 3 per cent of the total PF substance isolable from rabbit serum. It is insusceptible to mepyramine, to SBTI and to guinea-pig IPF.

The activation of a PF/Dil by dilution of rabbit serum was observed only twice in over 17 attempts and cannot be regarded as a characteristic property of rabbit serum.

A heat-stable PF is activated during fractionation of rabbit serum by the aqueous ethyl ether method ; it was found consistently associated with the α- and β-globulins. In moderate concentrations, it increases permeability within a few minutes ; the lowest effective concentrations do so after a lapse of several hours. Independently of the time of onset, increased permeability persists for at least 2–2½ hr., in sharp contrast to that induced by histamine or by rat and guinea-pig PF, which persists for only a few minutes. The long time-course is peculiar both to the PF and to rabbit tissue, since rabbit PF has a short time-course in the rat and guinea-pig, and both histamine and rat and guinea-pigs PF have a short time-course in the rabbit. Rabbit serum PF is moderately inhibited by SBTI, by guinea-pig IPF and by mepyramine, but does not liberate histamine from rat tissues ; it is not plasmin. Some 20,000 EBD can be isolated from 1 ml. serum.

As Paskhina (1956) observed, an IPF is present in the albumin fraction of rabbit serum. It is a slowly-acting and not very potent inhibitor of the serum PF.

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STUDIES ON THE MECHANISM OF SHOCK. THE INFLUENCE OF ENVIRONMENT ON THE CHANGES IN OXYGEN CONSUMPTION, TISSUE TEMPERATURE AND BLOOD FLOW PRODUCED BY LIMB ISCHAEMIA

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THE effect of a standardized ischaemic injury on the O_2 consumption, brain and liver temperatures and liver blood flow has been determined in the conscious rat under various environmental conditions. The influence of environmental temperature on the general response to injury (shock) has been described by Allen (1938) and others who have shown that an environmental temperature of about 20° is optimal for the survival of the unanaesthetized animal in ischaemic shock (Tabor and Rosenthal, 1947; Green and Stoner, 1950). When the air temperature is above or below this the survival time is reduced and mortality rate increased. This unexplained influence of the ambient air temperature is quite distinct from the effects of local cold or heat applied to the traumatized area (Allen, 1943). With the growing use of temperature control in surgery further knowledge of the effects of environmental temperature on the response to injury is desirable.

It is also important to decide whether changes in body temperature and O_2 consumption are an essential metabolic effect of injury or merely secondary to the circulatory and respiratory disturbance. The biochemical effects of haemorrhage have been mainly attributed to tissue anoxia but it is not certain that this is the whole explanation (Post, Visscher and Wiggers, 1948; Selkurt and Brecher, 1956) nor that it accounts for the changes in every tissue throughout the response (Blalock, 1944; Wilhelmi, Russell, Long and Engel, 1945; Hamrick and Myers, 1953; 1955). In ischaemic shock the situation is more uncertain for, while Tabor and Rosenthal (1947) consider that the fall in energy production is not due to a failure of O_2 transport some other effects of limb ischaemia are due to anoxia (Whiteley, Stoner and Threlfall, 1953; Stoner, 1955). However, for most of the survival period of rats in ischaemic shock at ordinary room temperatures (18–22°) the levels of energy-rich phosphates ($\sim$ P) in the liver, brain and uninjured muscle are normal, suggesting their adequate oxygenation before the terminal stages (Stoner and Threlfall, 1954; Threlfall and Stoner, 1957). Observations after limb ischaemia in the rabbit suggested that the early fall in the temperature of the liver and brain was not secondary to changes in blood flow (Stoner, 1954). These results were obtained with the internal calorimetry method (Grayson, 1952) and this method has been used again since it offers the most direct approach to these problems. A preliminary account of some of the results was given to the XXth International Physiological Congress (Stoner, 1956*a*).

METHODS

The experiments were performed on albino rats of the Porton strain (body wt. 290 ± 5 g.) fed on MRC diet 41 or 41*b* (Bruce and Parkes, 1949; 1956). Unless otherwise stated food and water were given *ad lib.* until the beginning of the experiment when the food was removed. Bilateral hind-limb ischaemia was produced with rubber tourniquets (Rosenthal, 1943) applied at about 10 a.m. during ether anaesthesia lasting about 3 min. The controls were given an ether anaesthetic of similar duration. The laboratory air temperature was between 18 and 22° (wet bulb 14–18°). Other environmental temperatures were obtained either in a Baird and Tatlock Humidity Control Cabinet set either at 30° or 37° (wet bulb 19° and 24° respectively) or in a cold room at 2–3°. For local heating the hind limbs were drawn into a small box with a heater element giving an air temperature of 31° in the box. Before the insertion of the thermocouples and blood-flow recorders the rats were kept in the animal house where the air temperature was similar to that in the laboratory.

Some rats were heat-acclimatized by keeping them in a hot room at 30° for 26–41 days (Beattie and Chambers, 1953). All operative procedures on these rats were carried out in the hot room.

Cold-acclimatization was obtained by keeping rats at 2–3° for 58–78 days (Blair and Dimitroff, 1956). The rats lost weight initially but after 2–3 weeks it increased until the 10th week when they began to lose weight again and their condition deteriorated. Rats showing this deterioration were not used and the average period of acclimatization was 66 days. Some were given 150 mg. ascorbic acid (neutralized) daily by subcutaneous injection (Dugal and Thérien, 1947; Dugal, 1954). These rats never lost weight and were in better condition than the untreated ones. However, as their responses to hind-limb ischaemia were not different, all the cold-acclimatized rats have been considered together. These rats were taken out of the cold room to insert the recorders and returned when they had recovered from the anaesthetic. This would not interfere with their acclimatization (Fregly, 1953).

Adrenal medullectomy was performed by the method of Evans (1936) as quoted by Ingle and Griffiths (1942). The rats were given 1 per cent (w/v) NaCl to drink for 1 week post-operatively and used 90–312 days later. The absence of the adrenal medulla was confirmed histologically.

Colonic temperature was recorded as before (Stoner, 1954). Rat brain is too small to assess its blood flow by internal calorimetry (Grayson, personal communication). Its temperature was determined with a copper-constantan ("Eureka") thermocouple (36 S.W.G.) inserted under ether anaesthesia into the left cerebral hemisphere through a small drill hole in the calvarium. Its position was checked at autopsy.

The liver temperature was measured and its blood flow assessed by internal calorimetry (Grayson, 1952) as previously described (Stoner, 1954; 1956*b*; 1957). Liver blood flow is expressed as the increase in thermal conductivity (δk) due to the flow of blood through the collar of liver around the recorder. The recorder was inserted, under ether anaesthesia, 1–3 days before the experiment. The position of the recorder in the liver was determined at autopsy and rats in which it was too near the surface or major vessels were excluded. The I^2/θ test(Grayson and Johnson, 1953) was sometimes used. The values obtained on the "dead" liver at the end of the experiments showed that the basal thermal conductivity of the liver was unaltered by limb ischaemia. A sufficient number of livers was studied histologically to show that limb ischaemia did not produce any structural alteration which would invalidate the use of the method.

At first the rats were given 50–100 mg. oxytetracycline hydrochloride (Terramycin-oral drops—Pfizer) by mouth when the recorders were inserted (Stoner, 1956*b*) as bacterial factors had not been incriminated in ischaemic shock in the rat (Koletsky and Gustafson, 1946). It was then found that although prophylactic oxytetracycline had no effect in experiments at 30°, at an environmental temperature of 18–22° it prolonged the survival time after a 4 hr. period of limb ischaemia without significantly affecting the ultimate mortality rate. After this partial confirmation of the results of Friedman, Schweinburg, Yasher and Fine (1957) the use of oxytetracycline was stopped. Thirty-one per cent of the controls and 28 per cent of the experimental rats received oxytetracycline. The treated rats were fairly evenly distributed in the different experimental classes. Since, apart from some prolongation of the

survival period, the reactions were unaltered by oxytetracycline the treated and untreated rats have been considered together.

The rats were placed in small wire cages (Stoner, 1956*b*) for the observations. At air temperatures of 30° and 37° they had to be kept in these cages throughout the experiment and it was often convenient to do so at 18–22°. These cages did not interfere with temperature regulation at environmental temperatures above 17°. At 2–3° possible "restraint hypothermia" was avoided by keeping the time spent in these cages to a minimum (<5 min.).

When fluid loss into the limbs after removal of the tourniquets is reduced by plaster casts the survival time is prolonged but the ultimate mortality rate is unaltered (Koletsky and Gustafson, 1950). The possibility that these small cages might have a similar effect was tested (Table I). The fall in colonic temperature was somewhat slower in the rats in small cages but the difference was not statistically significant. Some rats in the small cages survived longer but the final mortality was not significantly different in the 2 groups.

In some experiments the atmosphere breathed by the rat was controlled by putting a glass funnel in the cage so that it covered the rat's head and then either dry, CO_2-free air or O_2 was delivered to the funnel. The effect of atmospheric changes on the mortality rate after limb ischaemia was also tested. When the tourniquets were removed the rats were put in a gas exposure chamber containing a tray of soda-lime and either dry, CO_2-free air or O_2 was passed through it.

Blood pressure was recorded in rats lightly anaesthetized with ether by inserting a needle connected to a mercury manometer into the carotid artery.

Haemorrhage was produced in the anaesthetized rat by bleeding from the carotid artery into a reservoir (Johnson, 1954). Unanaesthetized rats were bled by the same method in which case the carotid cannula was inserted under ether anaesthesia some hours before the experiment or by cardiac puncture.

Oxygen consumption was measured by the method of Richards and Collison (1928). The roof of the chamber was modified to allow a recorder in the liver to be connected to the internal calorimetry apparatus. The procedure was as follows:

After an initial 30 min. recording period the rats were removed from the chambers and tourniquets applied under ether anaesthesia. The controls were also given ether. The rats were then kept for an hour at the same environmental temperature as the bath to allow the ether to be excreted. They were then returned to the chambers and further recordings made. To remove the tourniquets the chamber was opened and the rubber bands cut off as quickly as possible. By not removing the chamber from the bath and by keeping it closed as much as possible the equilibration time was shortened and recording could be recommenced after 10 min. (Moore, R. E., personal communication). The O_2 consumption was determined over successive 20–30 min. periods and expressed as ml. O_2 (S.T.P., dry) per kg. body wt. per hr. as the rats were of similar size and since O_2 consumption is thought to be a function of the muscle mass (Krebs, 1950).

In some experiments the air in the chambers was enriched with O_2 by flushing the chamber with a stream of O_2 when the rat was inside.

The O_2 content and capacity of portal vein and aortic blood was determined on 0·2 ml. samples by the method of Born, Dawes and Mott (1955) using Warburg manometers with solid-ended connections (Rodnight and McIlwain, 1954). The blood samples were withdrawn under light Na pentobarbital anaesthesia, into syringes moistened with a heparin solution and with the dead space filled with mercury. The blood was transferred to pipettes as described by Roughton and Scholander (1943).

As far as possible results have been expressed as the mean ± S.D. and a statistical comparison of the means made by Student's "*t*" test (Fisher, 1934). Where illustrations show the results in individual rats they have been chosen because they exhibit most of the features being described.

RESULTS

Control Observations on Tissue Temperature and Blood Flow

The initial (9–10 a.m.) temperature in the liver was 38·9° ± 0·6 and in the brain 38·1° ± 0·8. The effect of a short period (3 min.) of ether anaesthesia given at about 10 a.m. as in the tourniquet experiments was tested. Even such a short

anaesthesia caused a temporary fall in the liver and brain temperatures (Fig. 1).

The fall in liver temperature (Table II) was double that during the same period of the day in the fully conscious rat (Stoner, 1956*b*). When the environmental temperature was raised to 30° immediately after the anaesthesia the liver and brain temperature, after fluctuating for a short time, fell slowly but progressively during the day. The fall during the first 4 hr. was about the same as at the lower air temperature (Table II). A further rise in the environmental temperature to 37° affected the response to ether for all 3 rats showed a rise in the liver temperature (aver. 1·1°) at some time during the subsequent 4 hr. The brain temperature was not studied in these rats.

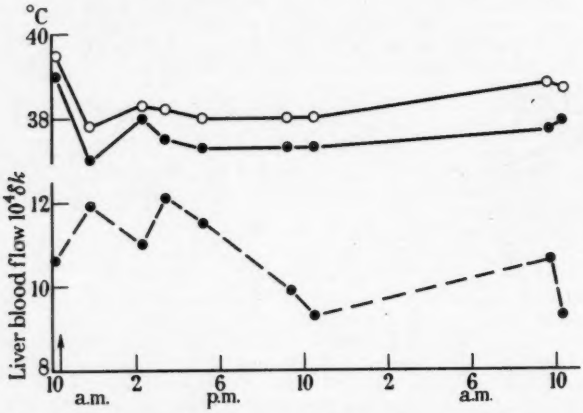

FIG. 1.—The effect of a 3 min. period of ether anaesthesia given at the arrow on the temperature of the liver (○———○) and brain (●———●) and on the liver blood flow (δk; ●- - -●) of a rat. Environmental temperature 19°.

When the environmental temperature was lowered to 2–3° care was taken that the rats had fully recovered consciousness before transferring them to the cold room. When this was done in 3 rats the liver temperature rose sharply by about 0·6°. The brain temperature, measured in one rat under these conditions fell gradually.

The results in the experiments on rats acclimatized to heat or cold made it unnecessary to determine the effect of anaesthesia alone in their case. The initial liver temperature (9–10 a.m.) in 5 rats acclimatized to 30° was 38·5° $\pm$ 0·3 and in 9 acclimatized to 2–3° was 39·3° $\pm$ 0·3. These values were not significantly different from those in rats at 18–22°.

At environmental temperatures of 18–22° and 30° the liver blood flow (δk) was unaffected by the short ether anaesthesia (Fig. 1; Table II). At 37° there was some indication of depression after anaesthesia (Table II) but the differences were not statistically significant. Lowering the environmental temperature to 2–3° did not affect liver blood flow. From these and previous experiments (Stoner, 1956*b*) it would seem that in an individual rat δk does not normally vary more than 20 per cent about its mean level.

Effect of Limb Ischaemia on Tissue Temperature and Liver Blood Flow

Environmental temperature 18–22°

During ischaemia.—Compared with the controls the maximum fall in the temperature of the liver and brain during a 4 hour period of limb ischaemia was greater but the difference was only significant ($P < 0{\cdot}001$) in the liver (Table III). The temperature of the liver and brain showed little recovery during the ischaemic period. In controls 4 hr. after anaesthesia the temperatures of the brain and liver showed some recovery in 66 and 56 per cent of the animals respectively. At the

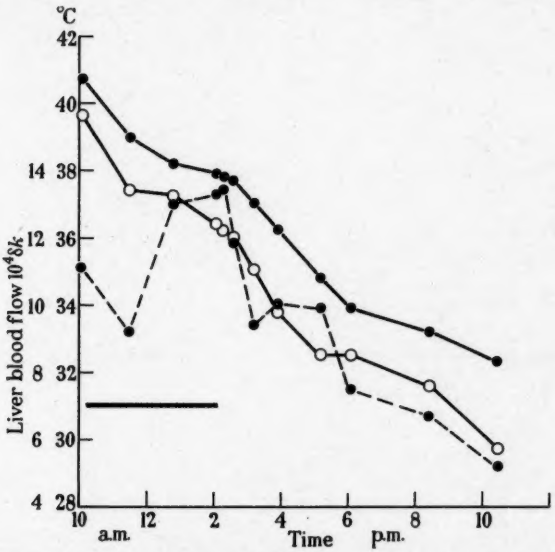

FIG. 2.—The effect of a 4 hr. period of bilateral hind-limb ischaemia (indicated by the horizontal line) on the temperature of the brain (○——○) and liver (●——●) and on the liver blood flow (δk; ●– – ●). The rat died before 9.0 a.m. on the following day. Environmental temperature 20°.

end of the 4 hr. period of limb ischaemia the corresponding percentages were 31 and 25. The liver blood flow did not alter significantly during the limb ischaemia (Table III).

After ischaemia.—At this environmental temperature a 2 hr. period of bilateral hind limb ischaemia was never fatal. A 4 hr. period led to death in 73 per cent of the rats, the average survival time being 18 ± 10 hr.

When the tourniquets were removed after 4 hr. there was a further fall in temperature (Fig. 2). This began almost immediately and in a fatal case continued until the rat died. At death the liver temperature was about 24°. The temperature in the brain fell more rapidly than in the liver and at about the same rate as in the colon (Table I). Heat loss was not exponential as in the dead animal (Stoner, 1954). The liver blood flow fell but soon stabilized at a level somewhat below the initial one. It was held at this new level for some time and then declined further, very

TABLE I.—*Mean Colonic Temperature in Two Groups of Rats after a 4 hr. Period of Bilateral Hind-limb Ischaemia*

In group A the rats were kept individually in large cages and in group B the rats were kept in the small cages used for observations on liver temperature and blood flow. Environmental temperature 18°. Number of rats shown in parentheses.

	Time (hr.) after ischaemia						
	0	1	3	7	9	11	13
	Colonic temperature °C. (mean ± S.D.)						
Group A	37·5 ± 0·8 (10)	35·6 ± 1·1 (10)	33·1 ± 1·3 (10)	29·2 ± 3·6 (10)	28·4 ± 3·9 (9)	26·7 ± 3·8 (8)	27·5 ± 4·2 (6)
Group B	37·2 ± 1·0 (10)	35·9 ± 1·1 (10)	34·1 ± 1·5 (10)	31·3 ± 3·0 (10)	31·3 ± 2·5 (9)	29·9 ± 2·9 (9)	28·2 ± 4·0 (9)

low values being obtained for some hours before death. The same pattern of changes was seen in 3 medullectomized rats.

Less marked changes occurred when the rats survived. The temperature in the liver (Fig. 4) and brain (Fig. 5) fell to between 32° and 34°. These low temperatures commonly persisted for about 36 hr. and even 48 hr. periods of this degree of hypothermia in the shocked rat are compatible with recovery. Death always occurred if the liver temperature fell below 30°. Liver blood flow was also reduced in the rats which recovered but not below 50 per cent of the initial value.

Fig. 3 shows the mean changes in liver temperature and δk during part of the survival period in 21 rats dying as a result of limb ischaemia and Fig. 4 shows the same in 12 survivors. The mean brain temperature in 8 fatal cases and 4 non-fatal ones is shown in Fig. 5. Assuming that the initial level of δk equals the mean level a variation of ± 20 per cent about this level could be expected in a normal rat. Applying this assumption to the rats which died (Fig. 3) it is seen that while the liver temperature fell steeply after removal of the tourniquets, δk did not fall appreciably below the normal range until after about 4 hr. In the survivors (Fig. 4) δk did not fall much below the normal range during the period of maximum change in liver temperature. The average fall in liver temperature in *all* these rats between the time of removing the tourniquets and the time when δk had fallen to 80 per cent of its initial value was 1·4°. In assessing these changes it should be borne in mind that the liver temperature is already depressed at the end of the period of limb ischaemia (Table III). The maintenance of a relatively normal hepatic blood flow for a time after removal of the tourniquets despite the decreasing blood volume may be related to the blood pressure level in these rats. At the end of the period of ischaemia it was slightly raised (about 120 mm. Hg). After removal of the tourniquets the blood pressure fell but soon became stable at about 70–80 mm. Hg. This level was maintained until shortly before the rat died. Thus for most of the survival period the arterial pressure is about the critical level (60–80 mm. Hg) above which the autoregulating mechanism of the liver maintains a constant blood flow (Johnson, 1954; Grayson, 1954; Ginsburg and Grayson, 1954; Grayson and Mendel, 1957).

The effects of a 2 hr. period of bilateral hind-limb ischaemia are relevant to this comparison of the changes in tissue temperature and blood flow. The arterial blood pressure is unaltered after this non-fatal period of ischaemia (Dexter, Stoner and Green, 1953) and no significant changes were found in liver blood flow (δk) during (Table III) or after it. Nevertheless, removal of the tourniquets was

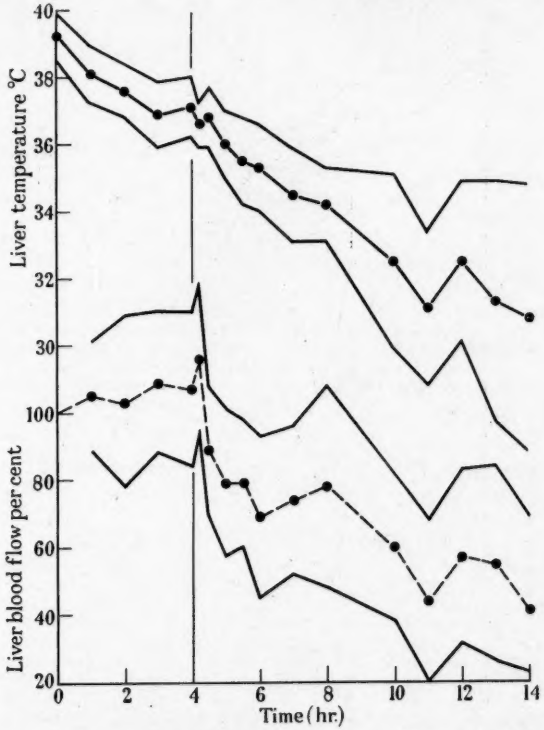

FIG. 3.—The mean liver temperature (●——●) and the mean liver blood flow (●- - -●) as a percentage of the initial value of δk in 21 rats dying some time after a 4 hr. period of bilateral hind-limb ischaemia. The removal of the tourniquets is indicated by the vertical line at 4 hr. The lines on each side of the curves of the means indicate ± S.D. Environmental temperature 18–22°.

followed by a further fall of 1·0° ±0·3 in liver temperature in the 4 rats studied. The temperature persisted at these levels (35°–37°) for a long time and normal levels were not regained for 24 hr. Brain temperature was measured in 3 of these rats and showed a further fall (0·9° ± 0·3) after removal of the tourniquets, recovering slowly as in the liver.

Environmental temperature 30°

Observations were made at this environmental temperature because it is the critical temperature for the fasted rat (Benedict and MacLeod, 1929; Swift and Forbes, 1939).

During ischaemia.—The maximum fall in brain temperature during the 4 hr. period of limb ischaemia was about the same as in the controls and in the experiments at an air temperature of 18–22° (Tables II, III). The liver temperature behaved differently (Table III). In the fed rat its change during the period of limb ischaemia was about the same as in the controls and significantly ($P < 0{\cdot}001$)

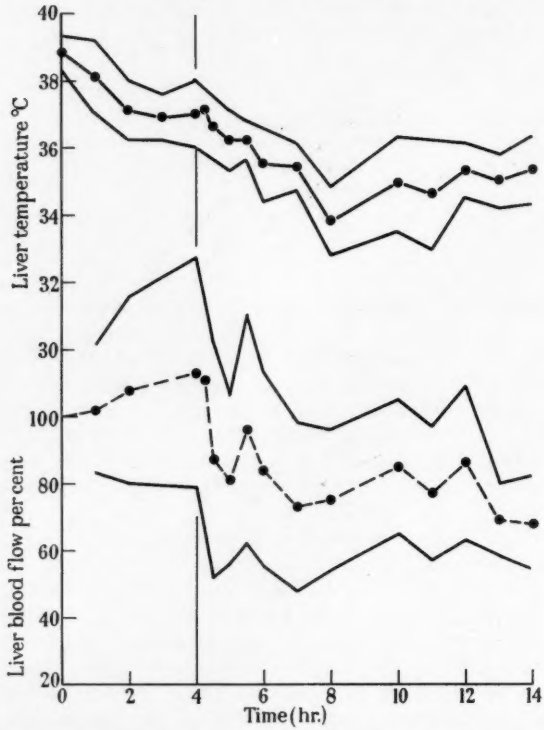

FIG. 4.—The mean liver temperature (●———●) and the mean liver blood flow (●– – –●) as a percentage of the initial value of δk in 12 rats which survived a 4 hr. period of bilateral hind-limb ischaemia. The removal of the tourniquets is indicated by the vertical line at 4 hr. The lines on each side of the curves of the means indicate ± S.D. Environmental temperature 18–22°.

less than in rats with limb ischaemia at an environmental temperature of 18–22°. In rats acclimatized to 30° the fall in liver temperature during limb ischaemia was not only less ($P < 0{\cdot}001$) than in the rats at the lower environmental temperature but also significantly ($P < 0{\cdot}05$) less than in the controls. The temperature of the liver and brain also tended to rise towards the end of the 4 hr. period of limb ischaemia without regaining the initial level. This was seen in 30 per cent of the experiments on brain temperature and in 46 per cent of those on liver temperature. The controls showed a steady decline in temperature during this period. When the rats were fasted overnight before the experiment the liver temperature rose

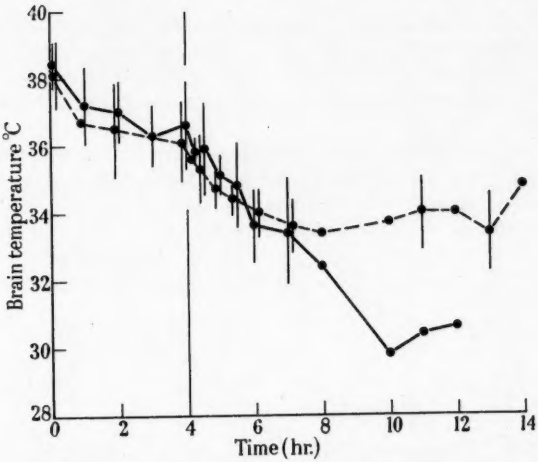

FIG. 5.—The mean brain temperature in 8 rats which died some time after a 4 hr. period of bilateral hind-limb ischaemia (●———●) and in 4 rats surviving this injury (●– – –●). The removal of the tourniquets is indicated by the vertical line at 4 hr. Where possible the S.D. of the mean has been calculated and is indicated by the vertical line through the mean point. Environmental temperature 18–22°.

TABLE II.—*The Effect of a Short (Approx. 3 min.) Ether Anaesthesia on the Brain and Liver temperature and the Liver Blood Flow (δk) in the Rat.*

Number of animals shown in parentheses.

Environmental temp. °C.	Fall in temp. (°C.) between 0 and 4 hr. after anaesthesia		Before anaesthesia	Liver blood flow (10^4 δk cal. cm.$^{-1}$sec.$^{-1}$ °C.$^{-1}$) 0–4 hr. after anaesthesia		
	Brain	Liver		Max.	Min.	Mean
18–22	1·3±0·8 (10)	1·2±0·7 (16)	10·8±2·9 (10)	12·8±2·6 (13)	10·6±2·5 (13)	11·3±2·3 (13)
30	1·2<0·4 (5)	1·3±0·6 (9)	10·9±3·1 (8)	11·2±3·2 (8)	9·8±3·1 (8)	10·5±3·2 (8)
37	—	See text	10·9±3·6 (3)	10·0±2·4 (3)	7·6±1·8 (3)	8·7±2·1 (3)

while the tourniquets were in place so that in all but one it was above the initial value at the end of the period of ischaemia (Table III). The liver blood flow fell ($P < 0{\cdot}05$) during the period of hind-limb ischaemia (Table III).

After ischaemia.—Raising the environmental temperature to 30° increased the mortality rate to 100 per cent and shortened the survival time to 1 hr. 53 min. ± 1 hr. 13 min.

When the tourniquets were removed the liver blood flow (δk) fell immediately and usually precipitously with little evidence of any stabilization (Fig. 6). There was an inverse relationship between the rate of fall of δk and the survival time in rats living more than 1 hr. after removal of the tourniquets. The best guide to the rat's condition and its proximity to death was the value of δk in the liver. At an

TABLE III.—*The Changes in Tissue Temperature and Liver Blood Flow (δk) during Bilateral Hind-limb Ischaemia in the Rat.*

Environmental temperature °C.	Duration of ischaemia (hr.)	Tissue temp. at end of ischaemia °C.		Av. max. change in tissue temp. during ischaemia °C.		Liver blood flow (10^4 δk cal. cm.$^{-1}$ sec.$^{-1}$ °C.$^{-1}$)			
		Brain	Liver	Brain	Liver	Before ischaemia	during ischaemia Max.	during ischaemia Min.	during ischaemia Mean
20	2	35·9±0·5 (3)	37·0±0·6 (4)	−1·7±0·9 (3)	−1·7±0·2 (4)	9·0±2·0 (4)	10·5±3·1 (4)	8·8±1·6 (4)	10·4±2·8 (4)
18–22	4	36.6±1·1 (17)	37·1±1·0 (46)	−1·8±1·0 (17)	−2·1±0·8 (46)	12·1±4·2 (42)	13·7±4.8 (42)	11·0±4·2 (42)	13·4±4·4 (42)
18–21 (Acclimatized to 2–3)	4	36·2 (2)	37·2±0·6 (8)	−0·9 (2)	−2·1±0·7 (8)	13·8±7·4 (8)	15·7±8·9 (8)	12·7±5·8 (8)	14·1±6·9 (8)
30	4	36·5±0·5 (7)	38·0±0·6 (20)	−1·2±0·5 (7)	−0·87±0·5 (20)	11·9±3·3 (20)	11·9±3·5 (20)	9·6±3·0 (20)	10·8±3·2 (20)
30*	4	—	38·5±0·4 (5)	—	−0·5±0·4 (5)	12·2±4·3 (5)	11·2±2·8 (5)	9·7±2·9 (5)	10·5±2·9 (5)
30 (Acclimatized to 30)	4	—	38·3±0·7 (4)	—	−0·5±0·4 (4)	9·1±0·3 (3)	8·4±0·4 (3)	6·8±1·1 (3)	7·6±0·9 (3)
30 (Medullectomized)	4	—	37·4±0·2 (6)	—	−1·4±0·3 (6)	7·5±2·3 (5)	7·4±2·4 (5)	5·8±2·3 (5)	6·6±2·2 (5)
37	4	—	40·1±0·5 (5)	—	+1·6±0·3 (5)	12·0±4·8 (5)	9·2±2·9 (5)	6·9±2·4 (5)	7·8±2·7 (5)
2–3	4	—	36·6±1·1 (3)	—	−1·9±1·0 (3)	7·4 (2)	9·9 (2)	9·2 (2)	9·5 (2)

Number of rats shown in parentheses. * fasting animals. The statistical significance of the changes is indicated in the text.

air temperature of 30° rats in ischaemic shock die very suddenly with little external warning. This is quite different from their behaviour at 18–22° where the best guide to their condition is the tissue temperature. The changes in liver blood flow were the same whether the rat was fasted or fed or acclimatized to 30° before the experiment.

The short survival period and precarious condition of these rats made it difficult to study the changes in arterial blood pressure. At the end of the ischaemic period the level was the same as in the rats at an air temperature of 18–22° but when the tourniquets were removed it rapidly fell below the critical level.

The most striking difference at this environmental temperature was the rise in liver temperature when the tourniquets were removed (Fig. 6). This began

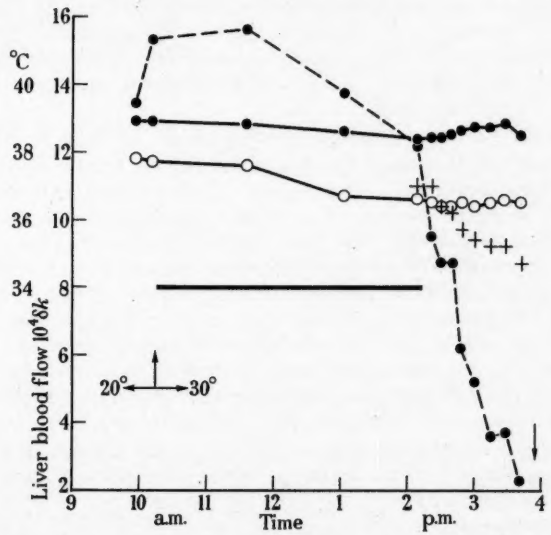

FIG. 6.—The effect of raising the environmental temperature to 30° on the responses of the liver (●———●), brain (○———○) and rectal (+) temperatures and of the liver blood flow (δk; ●– – –●) to a 4 hr. period of bilateral hind-limb ischaemia in a rat. The period of limb ischaemia is shown by the horizontal line and death by the arrow (↓).

almost immediately and continued until shortly before death when it fell abruptly. This increase which might be as much as 2·2°, was seen in all except 2 of 30 rats and in the 2 exceptions the survival time was under 45 min. The average rise above that at the end of the period of limb ischaemia was 0·6 ± 0·5 and this occurred in fed and fasted rats and in those acclimatized to 30°. In the fasted rats however, the liver temperature had risen during the period of limb ischaemia (Table III) so that in 80 per cent the maximum temperature after removal of the tourniquets was higher than that at the very beginning of the experiment. This only occurred in 38 per cent of the fed rats.

The response of the brain temperature in 8 fed rats was variable. In one it rose (0·8°) while in the others it fell slightly or remained relatively unchanged

(Fig. 6). The colonic temperature changes were similar to those in the liver but the temperature in the rectum fell (Fig. 6).

Local application of heat.—In 3 rats the hind limbs were kept in a box at 31° during and after the 4 hr. limb ischaemia while the air temperature around the rest of the animal was 20—22°. All died within 24 hr. of removing the tourniquets. The liver temperature and blood flow fell when the tourniquets were removed and the changes, compared with those in Fig. 3, were greater than when the whole rat was at an environmental temperature of 18–22°.

Effects of alterations in environmental temperature after limb ischaemia.—(*a*) Lowering the environmental temperature from 30° to 20–21°.

For 3 rats the environmental temperature, which had been 30° during the period of ischaemia, was lowered to 20–21° when the tourniquets were removed. All died within 12 hr. Their liver temperature and blood flow fell in the same way as in those kept at an air temperature of 18–22° throughout the experiment.

(*b*) Raising the environmental temperature from 20° to 30°.

This was observed in 8 rats. For 5 of them the air temperature was raised to 30° as soon as the tourniquets were removed. Three of these rats were killed 20 hr. later when they were recovering. The other 2 survived less than 12 hr. In all 5 the liver temperature rose after the period of limb ischaemia (Fig. 7) especially in those which recovered. The average maximum rise in liver temperature during the first 4 hr. after removal of the tourniquets was $2{\cdot}1° \pm 0{\cdot}3$. Under these conditions the hepatic blood flow (δk) fell more rapidly than in the rats at an environmental temperature of 20° for the whole of the experiment. The transient increase in δk in Fig. 7 immediately after the tourniquets were removed was seen occasionally in all the different groups of experiments and may be due to the release of adrenaline (Grayson and Johnson, 1953; Ginsburg and Grayson, 1954).

In 3 rats the liver temperature was allowed to fall after removing the tourniquets to between 30° and 35·3° before the environmental temperature was raised to 30°. All died within 9 hr. and the liver temperature rose steeply as soon as the environmental temperature was raised.

Adrenal medullectomy.—Six medullectomized rats were studied at an air temperature of 30°. They only survived 73 ± 13 min. after removal of the tourniquets but this was not significantly different from the survival time in the normal rats at this temperature because of the variation in the latter group. During the limb ischaemia the liver temperature fell significantly ($P < 0{\cdot}05$) more than in normal rats at this enviromental temperature and behaved more like that in rats at 18–22° (Table III). During this period δk did not alter significantly but after removal of the tourniquets it fell very steeply. Only 4 rats showed a rise in liver temperature after the period of limb ischaemia, the average maximum rise being $0{\cdot}2° \pm 0{\cdot}19$, significantly less ($P < 0{\cdot}05$) than in normal rats at this air temperature and also less ($P < 0{\cdot}05$) than in the rats at 37° although the survival time of the latter was even shorter.

Environmental temperature 37°

The effect of a further increase in the environmental temperature to 37° was observed in 6 rats.

During ischaemia.—The normal rat is unable to maintain its thermal equilibrium after a short ether anaesthesia at this air temperature (Table III) and when limb ischaemia was added to the strain of the high temperature the changes in tissue temperature and blood flow were more marked (Fig. 8; Table III). Two of the 6 rats died during the period of limb ischaemia.

After ischaemia.—The 4 rats which survived the 4 hr. period of limb ischaemia all died shortly (28 ± 11 min.) afterwards. Despite this short interval the already

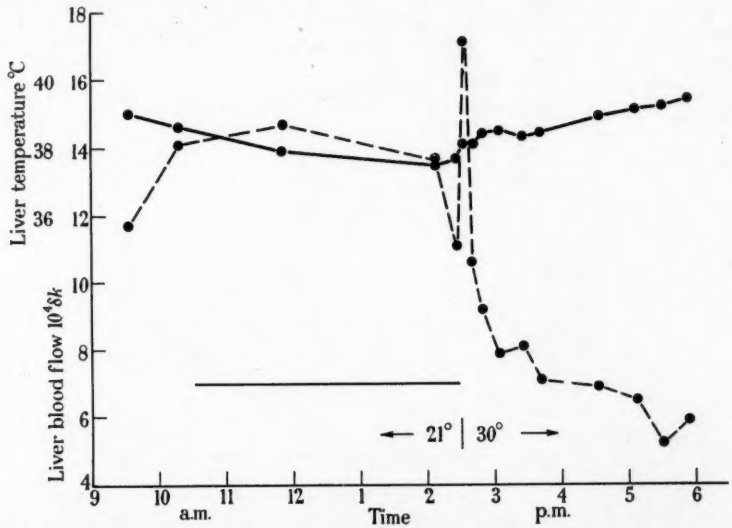

FIG. 7.—The effect of increasing the environmental temperature to 30° at the end of a 4 hr. period of bilateral hind-limb ischaemia at an environmental temperature of 21° on the response of the liver temperature (●———●) and blood flow (δk; ●– – –●) in a rat. The period of limb ischaemia is shown by the horizontal line.

elevated liver temperature continued to rise (0·6 ± 0·3) and there was also a rise in the brain temperature in the only experiment in which it was measured (Fig. 8). The hepatic blood flow fell precipitously when the tourniquets were removed.

Environmental temperature 2–3°

Effect of an environmental temperature of 2–3° during and after limb ischaemia.—Three rats were moved from an environmental temperature of 21° to the cold room (2–3°) as soon as the tourniquets had been applied and they had recovered from anaesthesia. Their behaviour during the period of limb ischaemia (Table III) was very similar to those kept at 18–22°. The rise in liver temperature usually seen when a rat is exposed to cold was not observed. All died within 20 hr. of removal of the tourniquets and the changes during that period were similar to those in rats at 18–22°. The liver temperature followed the lowest of the 3 temperature curves in Fig. 3 while the percentage change in δk was closer to the uppermost of the 3 curves in the lower part of that figure. The result was an

increase in the reduction of the liver temperature while its blood flow was within normal limits.

Effect of lowering the environmental temperature from 20–22° to 2–3° after limb ischaemia.—Eight rats were kept at 20–22° until they were moved to the cold room 21 min. to 2 hr. 36 min. after removal of the tourniquets. The liver temperature was then between 33·2° and 37·5°. All died within 20 hr. of removing the

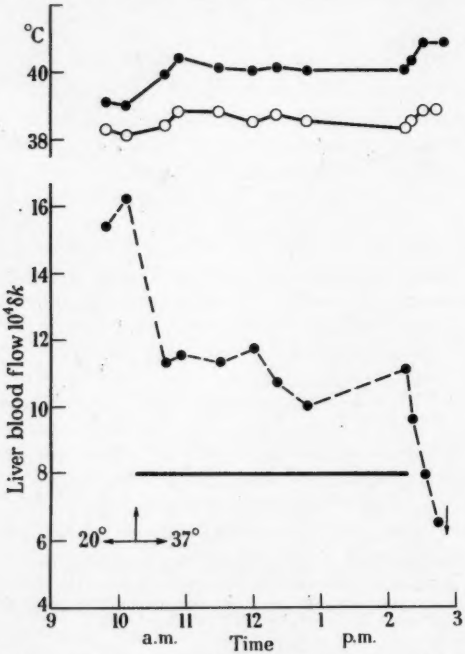

FIG. 8.—The effect of raising the environmental temperature to 37° on the responses of the liver (●———●) and brain (○———○) temperature and of the liver blood flow (δk; ●– – –●) to a 4 hr. period of bilateral hind-limb ischaemia in a rat. The period of limb ischaemia is shown by the horizontal line and death by the arrow (↓).

tourniquets and the effects of this change are shown in Fig. 9. The average rate of fall in the liver temperature after removal of the tourniquets was 0·18° ± 0·11 per 10 min. while the rats were at 20–22°. At 2–3° the rate was 0·83° ± 0·50 per 10 min., the difference being significant at $P < 0{\cdot}01$. Because of the rapidly changing temperature of the liver its blood flow was difficult to determine. It was clear, however, that this great fall in liver temperature was not accompanied by a comparable reduction in δk (Fig. 9).

The Behaviour of Cold-Acclimatized Rats

The increased resistance of the cold-acclimatized rats to ischaemic injury was first observed by Haist, Schachter, Sidlofsky, Hamilton and Baker (1956). We

have confirmed this and shown that the changes in muscle chemistry and temperature during and after ischaemia and the rise in the water content of the muscle after ischaemia are the same as in normal rats (Threlfall and Stoner, 1957). The mortality rate in 13 cold-acclimatized rats subjected to 4 hr. bilateral hind limb ischaemia at an environmental temperature of 18–22° was 23 per cent. None of

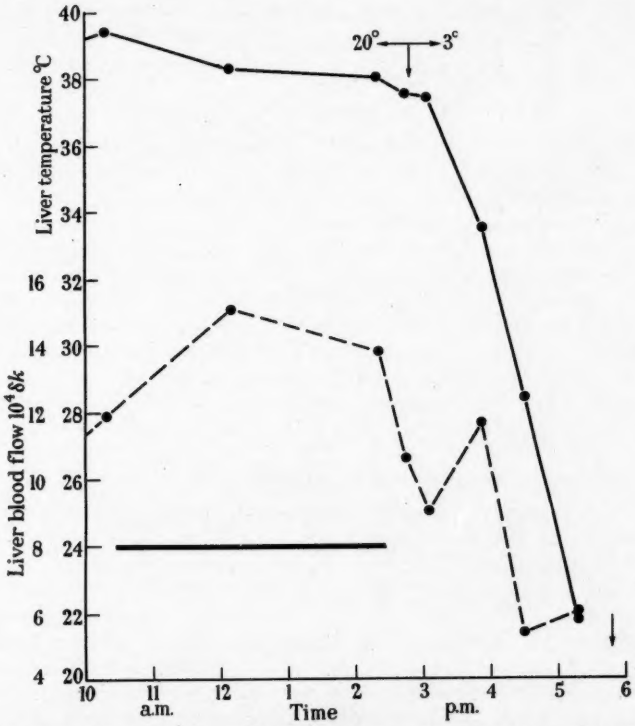

FIG. 9.—The effect of lowering the environmental temperature from 20° to 3° after a 4 hr. period of bilateral hind-limb ischaemia on the response of the liver temperature (●———●) and blood flow (δk; ●– – –●) in a rat. The period of limb ischaemia is shown by the horizontal line and death by the arrow (↓).

the 6 rats used to study the changes in tissue temperature and blood flow under these conditions died.

During ischaemia.—The effect of a 4 hr. period of limb ischaemia on the liver and brain of the cold-acclimatized rats was the same as in normal rats (Table III; Fig. 10).

After ischaemia.—The changes in liver temperature and blood flow after removal of the tourniquets are summarized in Fig. 10. Two points emerge when these results are compared with those in normal rats surviving this injury. The effect on δk during the first 5 hr. after release seems to be the same in the 2 groups

but the changes in liver temperature were very different. When the tourniquets were removed in the cold-acclimatized group the liver temperature showed no further fall for some hours. Only in 2 rats did the liver temperature fall to the levels seen in the surviving normal rats and then only after about 20 hr. In these 2 the fall in liver temperature to between 32° and 34° was not accompanied by a

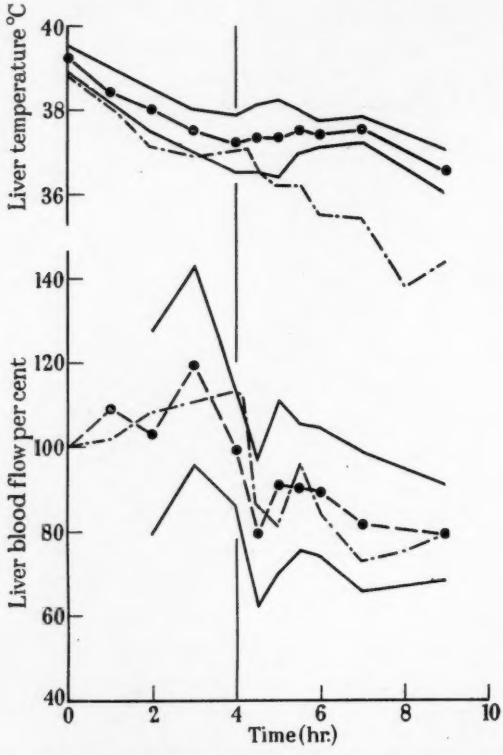

FIG. 10.—The mean liver temperature (●———●) and the mean liver blood flow (●- - -●) as a percentage of the initial value of δk in 6 cold acclimatized rats which survived a 4 hr. period of bilateral hind-limb ischaemia. The removal of the tourniquets is indicated by the vertical line at 4 hr. The lines on each side of the curves of the means indicate ± S.D. For comparison the mean changes in temperature and blood flow in normal rats surviving this injury (Fig. 4) are also shown (— . — . —). Environmental temperature 18–22°.

further reduction in δk which had remained stable at 70–75 per cent of the initial level for many hours. In the other rats the liver temperature had regained its normal level by 20 hr. after removal of the tourniquets although in most cases δk was still depressed. The brain temperature, measured in 2 cold-acclimatized rats, showed little further fall when the tourniquets were removed and normal levels were regained in 20 hr.

The remaining 3 cold-acclimatized rats were used as follows:,

In one the effect of a 4 hr. period of limb ischaemia was tested at an environmental temperature of 30°. Cold-acclimatization gave no protection. When the tourniquets were removed δk fell precipitously and the liver temperature rose 1·8° before the rat died after 1 hr. 44 min.

Two rats were returned to the cold room at 2° 2 hr. 15 min. after 24 hr. period of limb ischaemia at an environmental temperature of 18–20°. No change occurred in the liver temperature for about 40 min., then it began to fall. The rate of fall (0·26–0·33° per 10 min.) was very much less than in normal rats under these conditions and one rat was still alive 16 hr. later.

Effects of Haemorrhage

Acute haemorrhage was studied in 8 normal rats at an environmental temperature of 18–22° as it was thought that this might provide a useful comparison with the effects of limb ischaemia at 30°. In both cases hepatic blood flow can fall rapidly and the liver temperature rises after haemorrhage in the anaesthetized rabbit (Stoner, 1954). This latter was also seen by Johnson (personal communication) in the rat although not described in his paper (Johnson, 1954).

When rats were bled under pentobarbital anaesthesia there was a sudden rise in liver temperature of about 0·3°. However, this increase was short-lived and was soon replaced by a prolonged fall (Fig. 11). Furthermore, it only occurred in anaesthetized rats. In the unanaesthetized rat bleeding, either from an indwelling carotid cannula or by cardiac puncture, always lowered the liver temperature.

The Effect of Limb Ischaemia on Total Oxygen Consumption

The effect of a 4 hr. period of limb ischaemia on the total O_2 consumption of rats at an environmental temperature of 20° is shown in Fig. 12. During the limb ischaemia O_2 consumption was unaltered although more variable than in the controls. When the tourniquets were removed the O_2 consumption fell progressively until the rat died.

At an environmental temperature of 30° the initial O_2 consumption was reduced from 2070·8 $\pm$ 260·9 to 1434·4 $\pm$ 182·3 ml. O_2 kg. body wt. per hr. ($P < 0{\cdot}001$). Again, O_2 consumption was unchanged during the period of ischaemia (Fig. 12). When the tourniquets were removed the O_2 consumption fell immediately and at least as quickly as in the rats at an air temperature of 20°.

At an air temperature of 20° O_2 consumption reaches very low levels before the rat dies. The whole process occupies many hours and although all the rats used to construct curve 2 in Fig. 12 died, none did so during the period shown in that figure. The mortality rate of the 11 experimental rats of curve 4 shows that at an air temperature of 30° they died long before their O_2 consumption had fallen to the levels which were fatal at 20°.

It was not possible to obtain many simultaneous recordings of the liver temperature and blood flow. The few which were obtained showed that the changes were the same as described above.

Oxygen saturation of the blood

The changes in the O_2 saturation of the blood in the portal vein and aorta are shown in Table IV. There was a moderate fall in the percentage saturation of the

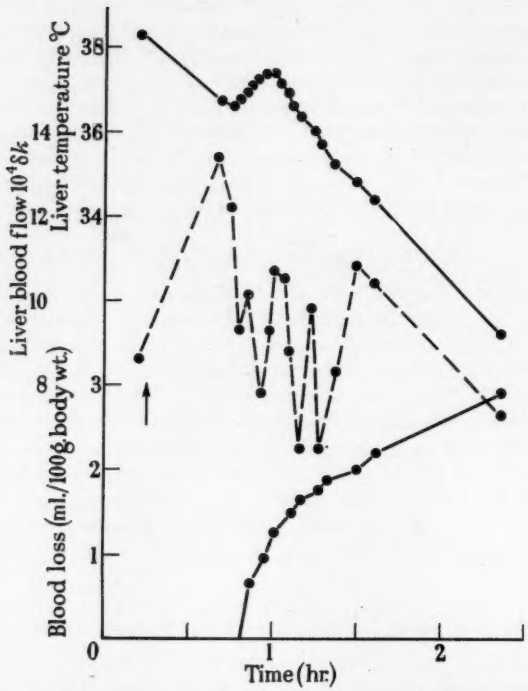

FIG. 11.—The effect of fatal haemorrhage on the liver temperature (●———●) and blood flow (δk; ●– – –●) in a rat anaesthetized with Na pentobarbitone at the arrow (↑). The volume of blood in the reservoir attached to the carotid artery is shown in the lowest curve. Environmental temperature 20°.

TABLE IV.—*Effect of a 4 hr. Period of Bilateral Hind-limb Ischaemia on the Percentage O_2 Saturation of the Blood in the Portal Vein and Aorta*

Number of rats shown in parentheses.

Air temp. °C.	Treatment	Percentage O_2 saturation	
		Portal vein	Aorta
18–22	Controls	60·3±11·4 (8)	94·1±3·3 (8)
18–22	1 hr. after ischaemia	45·4±6·3* (5)	—
18–22	4 hr. „ „	45·9±14·9* (10)	96·4±2·4 (8)
30	0·5 hr. „ „	39·4±9·6† (7)	93·3±0·5 (3)

* = significantly different from controls at $P < 0·05$.
† = significantly different from controls at $P < 0·01$.

portal blood at the first hour after removal of the tourniquets and this persisted unchanged for some time. This fall was accentuated when the environmental temperature was raised to 30°. The O_2 saturation of the arterial blood was unaltered at the times studied.

Effects of oxygen administration

Giving rats 100 per cent O_2 to breathe from the time of removal of the tourniquets did not decrease the mortality after a 4 hr. period of limb ischaemia (environmental temperature 22°). For technical reasons it was not possible to

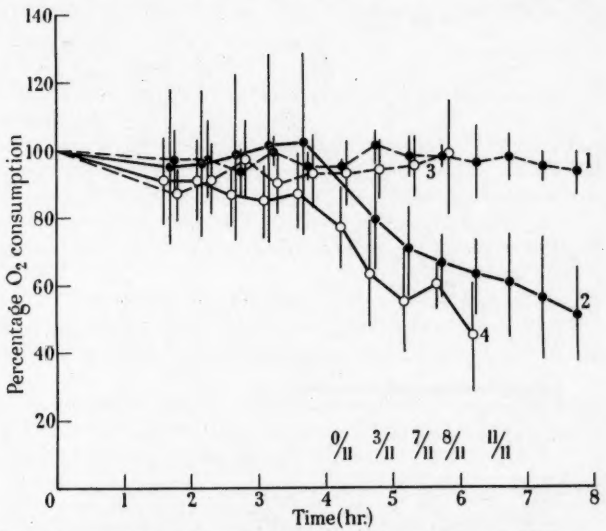

FIG. 12.—The mean percentage changes in O_2 consumption in rats subjected either to a short (3 min.) ether anaesthesia at 0 hr. or to bilateral hind-limb ischaemia between 0 and 4·0 hr. The vertical lines show the S.D. of the mean.

Curve 1 = 5 ether controls. Environmental temperature 20°.

Curve 2 = 5 rats with limb ischaemia. Environmental temperature 20°. All these rats died within 24 hr. of removing the tourniquets.

Curve 3 = 7 ether controls. Environmental temperature 30°.

Curve 4 = 11 rats with limb ischaemia. Environmental temperature 30°. All these rats died and their mortality rate is shown in the fractions at the bottom of the figure.

carry out similar experiments in the gas exposure chamber at an environmental temperature of 30°. The survival times in O_2 consumption experiments at 30° where the air in the chamber was enriched with O_2 when the tourniquets were removed were the same as in experiments carried out at 30° in air. This suggests that O_2 administration did not improve the condition of the rats at the higher environmental temperature.

The effect of enriching the air in the O_2 consumption chamber with O_2 at the time of removing the tourniquets was observed in 5 rats at 20° and in 6 rats at 30°. In both cases the O_2 consumption fell after the tourniquets were removed at the same rate as before.

A point which may affect the significance of the O_2 consumption figures obtained after the removal of the tourniquets in the experiments at an environmental temperature of 30° should be mentioned. To remove the tourniquets the lid of the chamber had to be removed and the rat momentarily lifted out so that the rat was exposed for a very short time to a lower air temperature. The temperature of the rat under these conditions is very labile. The liver temperature

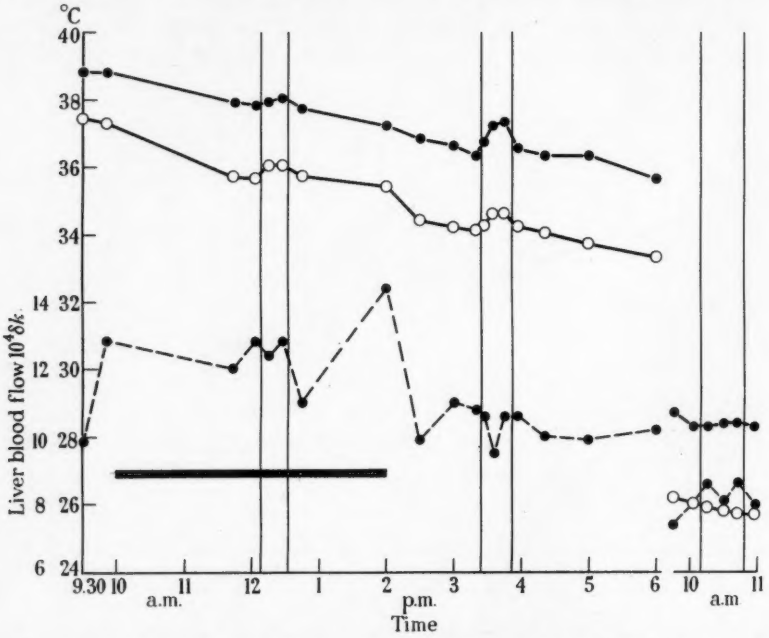

FIG. 13.—The effect of O_2 on the response of the liver (●———●) and brain (○———○) temperature and liver blood flow (δk; ●– – –●) to a 4 hr. period of bilateral hind-limb ischaemia in a rat. The period of limb ischaemia is shown by the horizontal line and O_2 was given between the vertical lines. Death occurred 2 hr. after the last observation. Environmental temperature 21°.

was found to fall by as much as 1·0° during this period and it was often some minutes before it recovered and showed its usual behaviour at this environmental temperature.

The effect of O_2 administration on tissue temperature and blood flow was studied in 4 control and 6 experimental rats at an environmental temperature of 18–22°. In the normal rat, changing the respiratory gas from air to O_2 for short periods (< 30 min.) had no effect on the temperature of the brain or liver or on the blood flow (δk) through the latter. When O_2 was given during the period of limb ischaemia the temperature of the liver and brain rose slightly in half the experiments, the most pronounced example is shown in Fig. 13. Hepatic blood flow was unaltered. When O_2 was given after removal of the tourniquets (Fig. 13) the temperature of the brain and liver rose abruptly provided their temperature

was above 30°. If their temperature was below this level the fall continued unaltered. The rise in temperature when it occurred was of the order shown in Fig. 13 and was well maintained while the O_2 was administered. When the rat was put back on air the temperature in the tissues rapidly returned to the initial level and continued to fall at the original rate.

DISCUSSION

The evidence in favour of the fall in liver and brain temperature during shock at an air temperature of 18–22° being a primary metabolic event and not secondary to the accompanying cardiovascular disturbance can be listed as follows:

Liver

1. The temperature falls during the period of limb ischaemia without change in the hepatic blood flow.
2. If the tourniquets are removed after 2 hr. there is a further fall in temperature although the hepatic blood flow and systemic blood pressure are unaltered.
3. If the tourniquets are removed after 4 hr. there is an immediate further fall in temperature although it is some hours before the systemic blood pressure falls below the critical level for autoregulation and hepatic blood flow is significantly reduced.
4. Experiments at other environmental temperatures show that the liver temperature can rise despite a much more severe reduction in blood flow and that the liver of the shocked rat is unable to respond to external cold like the normal rat.
5. In trauma-resistant cold-acclimatized rats the temperature changes after a 4 hr. period of limb ischaemia were minimal although the changes in blood flow were about the same as in normal rats.

Brain

The temperature fall during and after limb ischaemia was more severe than in the liver although the systemic blood presure was above the critical level (40 mm. Hg) for the autoregulation of the blood flow (Carlyle and Grayson, 1956) during most of the survival period.

To this evidence must be added that obtained in the rabbit (Stoner, 1954). There the temperature in the brain fell more rapidly than in the liver although its blood flow was better maintained. It was also found that the temperature of both organs fell below that of the mixed venous blood.

There are thus a number of reasons for believing that the fall in the temperature of the liver and brain during the *early part* of the response to limb ischaemia is unrelated to changes in their blood flow but this does not completely settle the question. The metabolism of an organ depends on the quality as well as the quantity of the blood supplied to it.

Soon after the tourniquets have been removed the O_2 saturation of the portal vein blood is reduced and normally this vessel contributes most of the blood going to the rat liver (Grayson and Mendel, 1957). However, the systemic blood pressure at this time is around 80 mm. Hg and the autoregulation of the hepatic blood flow will be occurring. This depends on the supply from the hepatic artery which is

maximal at this pressure when it can form 80 per cent of the total liver flow (Grayson and Mendel, 1957). As the O_2 saturation of the arterial blood is unaltered this should tend to counteract any effect of the change in the O_2 saturation of the portal vein blood. Even if most of the blood was still coming from the portal vein the change in its percentage O_2 saturation was not gross and it should be well within the capacity of the liver to compensate by increasing the extraction of O_2 (Hamrick and Myers, 1953; Selkurt and Brecher, 1956; Andrews and Glockling, 1956). The argument against anoxaemia seems even stronger in the case of the brain for the O_2 saturation of the arterial blood remains unaltered until the terminal phase and its blood flow is well maintained. It has been shown by Hirsch, Krinkel, Schneider and Schnellbäcker (1955) that by increasing the O_2 extraction rate the brain can maintain its O_2 consumption when the blood flow is only 50 per cent of the normal level and it can still do this in various forms of traumatic shock (Blalock, 1944). Nevertheless, although there is a case for excluding hypoxia as the cause of the temperature fall, the administration of O_2 (which will increase the tissue O_2 tension by raising the O_2 saturation of the portal vein blood (Wood, Mason and Blalock, 1940) and by increasing the amount of dissolved O_2) raised the temperature of the liver and brain during the early phase of shock. This question can only be resolved by direct determination of the O_2 tension in these tissues and work on this is in progress.

Although O_2 administration altered the tissue temperature the outcome of the injury was unaffected, as in the experiments of others (see Wiggers, 1950). This supports the view of Wayne, Barnes, Joyner, Tuttle, Lide and Green (1954) that maintaining the O_2 supply to the liver will delay but not prevent the onset of irreversible shock.

Regarding the mechanism of the decline in liver blood flow in shock these experiments support Johnson (1954) in that when the blood pressure is about the critical level autoregulation cannot be maintained indefinitely. Mesenteric pooling of the blood is often proposed as an important feature of shock (*e.g.*, Selkurt, Alexander and Patterson, 1947; Zanetti, 1952; Cull, Sibetta and Selkurt, 1956) but others have found little evidence of it (*e.g.*, Page and Abell, 1943; Reynell, Marks, Chidsey and Bradley, 1955). Recent work suggests that the explanation of the fall in liver blood flow should be sought in changes in the hepatic arterial supply (Grayson and Mendell, 1957). Autoregulation depends on this and the fall in blood volume after limb ischaemia together with the increased viscosity of the blood due to haemo-concentration and the fall in temperature (Brauer, Holloway and Leong, 1957) may prevent the hepatic artery from fulfilling this function in late ischaemic shock.

In assessing the importance of the changes in the liver circulation in ischaemic shock at an environmental temperature of 18–20° it may be significant that the change in liver temperature is a better prognostic guide than the change in liver blood flow. At the higher environmental temperatures liver blood flow was more severely affected and more closely related to the course of events. Further work is necessary before the more rapid fall in δk can be simply attributed to a greater and more rapid reduction in blood volume (and pressure) from the increased oedema in the injured legs (Threlfall and Stoner, 1957). Cardiac factors might also be involved.

A striking difference in the response to limb ischaemia at raised environmental temperatures was the increase in liver temperature when the tourniquets were

removed. The essential requirement for this effect is a high environmental temperature after the period of muscle ischaemia.

As the temperature of the liver is normally higher than that of the blood the liver temperature might be affected by variations in the amount of heat borne away by the blood passing through it. If this was suddenly reduced the temperature of the liver might rise although its metabolic rate remained unchanged. Johnson (personal communication) considered this a possible explanation of the rise after haemorrhage. Haemorrhage can certainly reduce hepatic blood flow but, in this laboratory, the rise in liver temperature was only seen in the anaesthetized animal and even then it was short-lived. The points against a physical explanation of this phenomenon may be summarized as follows:

1. At an air temperature of 37° the rise in liver temperature began during the period of limb ischaemia before any gross change in δk.

2. The effect was greater in fasted than fed rats although the vascular changes were similar.

3. The effect was not directly related to the severity of the fall in hepatic blood flow. It was most striking when the air temperature was not raised until after the period of limb ischaemia, δk then fell rapidly but not so quickly as when the environmental temperature was high throughout.

4. Heat loss through the lungs is a complex question but it seems agreed that the amount of heat lost in this way is very small, if any, even when breathing cold (− 35°) air (Mather, Nahas and Hemingway, 1953; Good and Sellars, 1957). It is unlikely, therefore, that a rise in the air temperature from 20° to 30° would significantly inhibit the diffusion of heat through the diaphragm. Experiments with thermocouples in the liver, cephalic end of the trachea and thorax just above the diaphragm shown that this rise in environmental temperature had no effect on the temperature gradient across the diaphragm although the temperature of the air in the trachea rose slightly.

Although the rise in liver temperature is probably related to metabolic events little can be said about its mechanism. The sudden rise followed by an equally sudden fall just before death is like the change produced by 3,5-dinitro-*o*-cresol (Stoner, 1956*b*). Impaired mitochondrial function in haemorrhagic shock has been reported by Strawitz and Hift (1956*a*, *b*). Dr. W. N. Aldridge has kindly tested the liver mitochondria from rats at an environmental temperature of 30° killed 30–50 min. after a 4 hr. period of limb ischaemia. Their behaviour in his system (Aldridge, 1957) was normal both with regard to O_2 uptake and phosphorylation. Adrenal medullectomy inhibited this rise in the liver temperature at 30°. This cannot be entirely explained by the short survival time as the rise was less than in rats at 37° where the survival time was even shorter. This implicates adrenaline as a factor in this response (cf. Gyermek, 1950) but obviously it is not essential.

The fall in O_2 consumption after injury at ordinary environmental temperatures has long been known (Aub, 1920) but the results at 30° were unexpected since, in mice, Tabot (personal communication) found that the fall was less at the higher environmental temperature. With the rise in liver temperature at this air temperature it was thought that O_2 consumption might be raised for a time after removal of the tourniquets. This was never seen. The technical difficulty of these measurements has been mentioned but it is doubtful if a short phase of increased consumption was lost in that initial period of temperature instability. At an air temperature of 20° the fall in O_2 consumption accorded with the clinical state. At

30° O_2 consumption fell similarly but death suddenly intervened while it was still at a relatively high level as if the changes affecting O_2 consumption were distinct from those causing death.

According to Krebs (1950) most of the O_2 consumed by an animal is used by its muscle. If the changes after limb ischaemia are due to alterations in muscle metabolism are they primary or secondary to a fall in O_2 transport ? The answer may vary with the environmental temperature. In dogs and rats (Bigelow, Lindsay, Harrison, Gordon and Greenwood, 1950; Hegnauer and D'Amato, 1954; Bartlett, Bohr and Inman, 1955) when the body temperature falls without shivering O_2 consumption falls linearly with the temperature, the Q_{10} being approximately 2. If the general level of muscle temperature is equated with that in the colon (Table I) the fall in O_2 consumption after limb ischaemia at an environmental temperature of 20° is what would be expected from the fall in temperature. Oxygenation of the muscle would be adequate and this is confirmed by the persistence of a normal nucleotide pattern in the uninjured muscle practically until death (Stoner and Threlfall, 1954; Threlfall and Stoner, 1957). However, the temperature of the rat is easily depressed by hypoxia (Goëbel and Klante, 1953) so that the decision as to which is the initial change, the lowering of muscle metabolism or the fall in O_2 consumption, awaits the determination of the muscle O_2 tension. At an air temperature of 30° where the fall in muscle temperature is prevented the muscle chemistry indicates an inadequate O_2 supply (Threlfall and Stoner, 1957) and reduced O_2 transport may be a factor in the fall in O_2 consumption.

Exposure to cold throughout the experiment reduced the local effect of the injury and this may explain the slower fall in hepatic blood flow under these conditions. The increased dichotomy of the temperature and blood flow changes and the absence of the normal response to cold both in these rats and those exposed after removal of the tourniquets indicates that not only is liver metabolism depressed in shock but that this depression occurs before significant changes in blood flow.

The precipitous fall in tissue temperature on exposure to cold after removal of the tourniquets shows the lack of temperature control in shock. Although it is difficult to apply observations of this sort directly to man (Green and Stoner, 1954) it would seem that experimental work on the application of hypothermia in surgery should not be confined to the cooling of normal animals. The results obtained at an environmental temperature of 18–22° support a previous conclusion (Stoner and Threlfall, 1954) that for much of the shock period there is a balanced reduction in the utilization and formation of $\sim$P. It is only terminally that the blood flow through the liver is inadequate to support the formation of $\sim$P in sufficient amount to meet its requirements and the $\sim$P level falls. It was thought (Stoner and Threlfall, 1954) that raising the environmental temperature to 30° merely prevented the fall in tissue temperature and preserved the demand for energy above the level which could be met so that the $\sim$P level fell and the rat might be said to die of "energy depletion". This may be correct for the brain and muscle but the situation in the liver is more complicated. The temperature of the liver is not only maintained, it increases, raising the demand for energy at a time when the liver blood flow is falling rapidly. It is tempting to equate the rise in temperature with a disorganized attempt to produce the energy for its needs and the final temperature fall with exhaustion of the sources of energy. While these

results raise many unsolved problems they emphasize the importance of environmental factors in the response of the body to injury.

SUMMARY

The effect of limb ischaemia on the temperature in the brain, liver and colon and on the liver blood flow has been studied at various environmental temperatures in normal and heat- and cold-acclimatized rats and rats after adrenal medullectomy. Its effect on the arterial blood pressure and the O_2 saturation of the blood in the portal vein and aorta was examined in normal rats.

At an environmental temperature of 18–20° the temperature of the liver and brain fell during the period of limb ischaemia and continued to fall afterwards. The extent of the changes depended on the duration of the limb ischaemia. The liver blood flow, unaffected by a 2 hr. period of limb ischaemia, was reduced some hours after a 4 hr. period. After the latter the arterial blood pressure fell and remained at about the critical level for autoregulation of the hepatic blood flow until the rat was moribund. In the cold-acclimatized rats liver and brain temperatures were unaffected by a 4 hr. period of limb ischaemia although the changes in hepatic blood flow were the same as in normal rats.

Raising the environmental temperature accelerated the fall in blood pressure and hepatic blood flow after limb ischaemia but the liver temperature rose. Lowering the air temperature accentuated the fall in tissue temperature without altering the changes in liver blood flow after limb ischaemia.

The liver temperature rose after acute haemorrhage provided the rat was anaesthetized, otherwise it fell.

The O_2 saturation of the portal vein blood fell after limb ischaemia.

Total O_2 consumption was unchanged during limb ischaemia but fell when the tourniquets were removed at air temperatures of 20° and 30°.

O_2 administration did not alter the survival time or mortality rate after limb ischaemia or the rate of fall in O_2 consumption. It did, however, cause a rise in liver and brain temperature under certain conditions.

Some further investigation of these phenomena is described and the results are considered from the point of view of the effect of environment on the response to injury and with regard to the question whether the changes in tissue temperature and O_2 consumption represent primary metabolic changes or are secondary to the cardiovascular disturbance.

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THE EFFECTS OF LOSS OF DIFFERENT VIRULENCE DETERMINANTS ON THE VIRULENCE AND IMMUNOGENICITY OF STRAINS OF *PASTEURELLA PESTIS*

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PAST studies on immunity to experimental plague were conducted in the absence of knowledge of certain factors which determine virulence in the causative organism *Pasteurella pestis*. The main result of such studies has been to establish conclusively that an antigen, intimately associated with the envelope which surrounds organisms under suitable cultural conditions, plays a primary role in the protection of mice, rats, monkeys and probably man against virulent challenge (Meyer, 1948). This antigen retains its antigenicity and protective action for these species following its extraction and purification from acetone-killed dried organisms. It has variously been termed envelope substance by Rowland (1914), Schütze (1932) and Bhatnagar (1940), capsular antigen (Amies, 1951) fraction 1 (Baker, Sommer, Foster, Meyer and Meyer, 1952) antigen A by Seal (1951) and antigen 3 by Crumpton and Davies (1956). Since the relation of this antigen to immunity has been investigated most thoroughly by Meyer's school we will refer to it as fraction 1 (F1) throughout this paper. In contrast to its high immunogenicity in the above species, in its extracted form it is reported to play only a minor rôle in the protection of guinea-pigs (Baker *et al.*, 1952). In this animal high protection appears to be associated with the insoluble material remaining after saline extraction of acetone-treated organisms or with a soluble antigenic complex which can be obtained under certain cultural conditions (Englesberg and Levy, 1954).

Using organisms killed by a variety of treatments, or materials extracted from them, no relationship appeared to exist between the immunogenicity of the products and the virulence of the strains from which they were derived, when tested in any animal species. There is, however, general agreement that living vaccines confer an immunity superior to that given by equal or multiple amounts of the corresponding killed vaccines—a superiority which has been variously ascribed to the retention of unaltered antigens in the live state (Otten, 1936) or to the persistence and multiplication of the live organism *in vivo* (Meyer, 1948). Girard and Robic (1936) attributed the success of their EV76 strain, which has been used as a live vaccine for large scale inoculation of man in Madagascar, to its retention of a residual degree of virulence, and regard the Tjiwidej strain similarly employed by Otten in Java to be somewhat more completely attenuated than their own. In more recent times emphasis has been placed on the use of purified, non-toxic F1 as the material of choice for the immunization of the mouse, monkey and man against plague and convincing evidence has been presented in support of its value (Meyer and Foster, 1948).

The new knowledge of the basis of virulence in *Past. pestis* which has accumulated during the past few years permits a more rational approach to the problem of plague prophylaxis. This knowledge may be briefly summarised as follows. For full virulence (*i.e.*, to have an intraperitoneal or subcutaneous A.L.D. of 10 organisms, or less) in both mice and guinea-pigs a strain must, besides presumably being toxigenic, be capable of elaborating envelope antigen (F1+), produce V and W antigens (VW+) and form densely pigmented colonies on defined media containing haemin (P+); *i.e.*, strains which show full virulence for both species are all F1+ VW+ P+ (Burrows, 1957). The loss of ability to produce F1 (*i.e.*, F1−), while retaining the characters VW+ P+, still permits a strain to show full virulence for mice but reduces its virulence for guinea-pigs (A.L.D. *ca.* 10^5 organisms, Burrows, 1957). Strains lacking only the ability to produce V and W antigens, *i.e.*, those which are F1+ VW− P+, are avirulent for both species, (A.L.D. mice *ca.* 10^8 guinea-pig $>10^{10}$). The loss of ability to produce pigmented colonies (F1+ VW+ P−) results in reduced virulence for mice (A.L.D. poorly defined over the range 10^3–10^6) and great loss of virulence for guinea-pigs (A.L.D. *ca.* 10^{10}). The virulence of such strains for mice but not for guinea-pigs, can be fully restored by the simultaneous injection of minute, non-toxic, amounts of iron salts (Jackson and Burrows, 1956*b*). With this information available it is understandable that the two strains widely used as live vaccines in man could show somewhat different properties with respect to immunization against experimental plague; both are avirulent but for different reasons. Tjiwidej S is F1+VW −P+, EV 76 is F1+VW+ P−. It seemed very desirable to assess systematically the comparative virulence and immunogenicity of strains showing different combinations of the characters at present recognized as being of importance for the manifestation of virulence. To do so it was necessary that such strains should in all other respects be as genetically identical as possible. For studies on virulence we accordingly selected from a fully virulent FI + VW + P + strain a series of derivatives lacking any one, two or all of these three individual virulence determinants. For comparative immunogenicity studies we ideally required strains which could be used in the living state, without resort to therapeutic treatments, and which would not introduce ambiguities resulting from possible differential multiplication of different vaccine strains *in vivo*. These ideals were achieved by the use of a series of purine dependent strains, selected from the same virulent parent and corresponding in other characters with the series derived for comparative virulence studies. Such strains appear incapable of growth in mice and guinea-pigs through the non-availability of their specific nutritional requirement (Bacon, Burrows and Yates, 1951; Burrows, 1955; Garber Hackett and Franklin, 1952). We have used these vaccine strains to assess the extent to which the different virulence determinants contribute towards active immunity in mice and guinea-pigs, the antigenicity of vaccines in mice, guinea-pigs and rabbits, and their abilities to produce in guinea-pigs and rabbits antisera which have passive protective action for mice.

MATERIALS AND METHODS

Strains.—The fully virulent strain MP6 used in several other studies (Burrows and Bacon, 1954*a*, *b*; 1956) was taken as the parent strain from which mutants having all permutations of the three virulence characters F1, VW and P were selected by methods described below. They were all purine independent. From the purine dependent mutant M1, which was

derived from MP6, a corresponding series of mutants was selected, all of which were, of course, similarly purine dependent. For brevity we will henceforth refer to strains in terms of their virulence characters, *e.g.*, F1−VW+P− (or simply −+−) describes a strain which does not produce F1 antigen, fails to form pigmented colonies but still produces V and W antigens. The character of purine dependence or independence will be referred to as necessary. The lineage of the mutant strains, together with their virulence characters, is shown in Table I.

TABLE I.—*Lineage of Derived Strains*

	Parent Strain	F1	VW	P	Mutant : Parent → Strain	F1	VW	P	Mutant Strain	F1	VW	P
Purine independent	MP6	+	+	+	M14	+	+	−				
	,,				M17	−	+	+	M18	−	+	−
	,,				M15	+	−	+	M32	+	−	−
	,,				M16	−	−	+	M33	−	−	−
Purine dependent	M1	+	+	+	M29	+	+	−				
	,,				M22A	−	+	+	M26A	−	+	−
	,,				M20	+	−	+	M21	+	−	−
	M20	+	−	+	M27	−	−	+	M28	−	−	−

Selection of strains deficient in virulence characters.—To select for F1−, advantage was taken of the ability of F1+ antisera compactly to agglutinate F1+ organisms. Suspensions of F1+ organisms, grown at 37° for 24 hr. on tryptic meat agar (TMA) slopes, were inoculated to a density of *ca.* 5×10^8 organisms per ml. into 2 ml. of liquid medium consisting of equal volumes of tryptic meat broth (TMB) and rabbit antiserum containing abundant F1 antibody. The cultures were incubated static overnight at 37° by which time all organisms had sedimented leaving clear supernatants. They were then vigorously shaken by hand, allowed to settle for 1 hr., 0·1 ml. of the slightly turbid supernatant transferred to fresh antiserum medium and the above processes repeated. Between each transfer a small aliquot of slightly turbid supernatant was streaked on TMA to give well isolated colonies. About 20 of these were individually tested for ability to produce F1 by growing them on TMA slopes at 37° for 24 hr. and diffusing the growths against F1 antibody by the technique of Ouchterlony (1953). If none of the tested strains was F1− the process of serum selection was continued until successful. In most cases the required F1− derivative was obtained within 3 cycles of selection.

Initial attempts to select VW− strains were conducted as for F1− above, but using F1−VW+ antisera; however, it was subsequently found that such strains were selected equally readily by the method used for F1− selection. They would probably be obtained without difficulty by any single colony selection. Single colony isolates were incubated with rotation in TMB pH 7 for 6 hr. at 37° and small volumes of the whole cultures diffused against antisera rendered specific for V and W antigens by appropriate absorption (Burrows and Bacon, 1956). Cultures failing to produce demonstrable V and W antigens were retained.

Selection for P− was effected by spot inoculating the corresponding P+ strain on pigmentation agar (Jackson and Burrows, 1956*a*) and culturing from the non-pigmented sectors which arose on prolonged incubation at 28°.

Failure to produce F1 and VW antigens was additionally checked *in vivo* by injecting *ca.* 5×10^6 organisms i.p. into mice (together with hypoxanthine for purine deficient strains) and observing India ink preparations of the peritoneal contents 6 hr. later. Under these conditions F1+ strains showed each organism to be surrounded by a clear halo which was entirely absent from F1− organisms. The former were rapidly and compactly agglutinated by F1 specific antiserum while the latter were unaffected. Inspection of the phagocytosis of organisms by polymorphonuclear leucocytes in stained preparations of the peritoneal contents confirmed the VW nature of the strains; low ingestion indicated VW+, high ingestion VW−.

Vaccines.—Purine dependent strains in the living state were used throughout except where otherwise stated. To ensure the presence of preformed antigens in the vaccines before injection (since these strains have limited metabolism *in vivo*) all vaccines were grown at 37° on TMA slopes for 16 hr. (to ensure the presence of F1 antigen in F1+ strains) and were

then rotated, at a density of 2×10^8 organisms per ml., in TMB pH7 at 37° for 4 hr., similarly to ensure the presence of V and W antigens. The vaccines were freshly prepared for each dose and checked for conformity with their virulence characters. All groups of animals were injected on the same days and with the same batch of vaccines. Mice were injected i.p. with an initial dose of 10^7 organisms, followed later by doses of 10^7 organisms at 7 day intervals; guinea-pigs and rabbits similarly received 10^6 organisms subcutaneously followed by doses of 10^8. A dose of 10^6 organisms is approximately equivalent to 1 μg. of dried organisms.

Antisera.—Antisera were collected at the intervals during immunization stated in the text. Individual sera from groups of 10 mice, 5 guinea-pigs or 3 rabbits were pooled before use.

Active protection tests.—Mice, immunised over periods stated in the text, were challenged i.p. with 2000 virulent MP6 organisms rendered resistent to phagocytosis by incubation in TMB for 4 hr. at 37° with rotation. Guinea-pigs were challenged wth 1 million similar organisms injected subcutaneously. For some experiments the F1−VW+P+ strain M23, similarly prepared and in the same doses, was used as challenge organism.

Passive protection tests.—Antisera produced in guinea-pigs and rabbits were tested for ability to protect mice passively against similar challenge with MP6, or M23, to that used in active protection tests. The antisera (0·2 ml.) were injected i.p. and the challenge dose given i.p. 1 hr. later. To express active or passive protection abilities of vaccines or antisera by a single figure which, as introduced by Meyer (Meyer and Foster, 1948) accommodates both the percentage of deaths and the times of survival of challenged animals, we have calculated protection indices thus:

$$\text{P.I.} = \frac{\text{Mean time of death in days}}{\text{Per cent deaths}} \times 100$$

Our protection index is thus 100 times the reciprocal of that used by Meyer and has the advantage, we think, of bearing a direct relationship to the degree of protection, in contrast to the somewhat confusing inverse relationship of Meyer's mouse protection index.

In both active and passive protection tests, animals were observed for a minimum of 21 days.

Assays of antigens and antibodies.—The technique we devised for F1, V and W antigen assays, using an agar diffusion precipitin method, was subsequently applied by Thorne and Belton to the assay of anthrax antigens and antibodies and a detailed description of the full procedure can be found in their publication (Thorne and Belton, 1957). We assayed F1, V and W antibodies by diffusing 0·1 ml. of serial 1 in 2 dilutions of antiserum against "standard" preparations of the corresponding antigens and observed the highest dilution which still gave a visible specific line of precipitation. Since our V and W "standards" were known to contain other antigens it was necessary to absorb antisera with VW− organisms before estimating their V and W antibody contents. For this absorption we used 50 mg. of acetone dried TS organisms per ml. of antiserum throughout.

RESULTS

Virulence of the derived strains

It is evident that the genetic determinants of the characters F1, VW and P are autonomous, permitting the loss of any one character without affecting the expression of the others. In no instance has a selected strain shown reversion from a "−" character to the corresponding "+" state. Although purposely sought we did not isolate any strains having the ability to produce V antigen but not W antigen, or *vice versa*; neither have we encountered such in the wide variety of strains, from many geographical sources, which we have examined. The separate rôles of these two antigens in virulence and immunogenicity phenomena cannot therefore be assessed until purified V and W antigens are available. Their invariable association suggests that they may have a common precursor.

TABLE II.—*Virulence of Strains Deficient in Virulence Characters*

Purine independent strains						Purine dependent trains					
				A.L.D.						A.L.D.	
	F1	VW	P	Mice	Guinea-pigs		F1	VW	P	Mice	Guinea-pigs
M32	+	−	−	$1–2\times10^8$	$>10^{10}$	M21	+	−	−	$4–8\times10^8$	$>10^{10}$
M15	+	−	+	$1–2\times10^8$	$>10^{10}$	M20	+	−	+	$4–8\times10^8$	$>10^{10}$
M14	+	+	−	$10^3–10^7$	$>10^{10}$	M29	+	+	−	$2–4\times10^8$	$>10^{10}$
MP6	+	+	+	<10	<10	M1	+	+	+	$2–4\times10^8$	$>10^{10}$
M18	−	+	−	$10^4–10^7$	$>10^{10}$	M26A	−	+	−	$2–4\times10^8$	$>10^{10}$
M17	−	+	+	<10	$10^3–10^6$	M22A	−	+	+	$4–8\times10^8$	$>10^{10}$
M33	−	−	−	$1–2\times10^8$	$>10^{10}$	M28	−	−	−	$4–8\times10^8$	$>10^{10}$
M16	−	−	+	$2–4\times10^8$	$>10^{10}$	M27	−	−	+	$2–4\times10^8$	$>10^{10}$

Organisms injected intraperitoneally suspended in 0·2 ml. buffer.

The results presented in Table II illustrate, in terms of their average lethal doses, the virulence for mice and for guinea-pigs of the 15 derivatives of the virulent strain MP6. These results, obtained with strains having the greatest possible genetic similarity, substantiated our previous conclusions regarding virulence determinants in *Past. pestis*. The virulence of strains having two or more deficiencies accorded completely with predictions based on the effects of loss of individual characters.

Purine independent strains were injected subcutaneously into mice and guinea-pigs in various combinations such that all animals received organisms bearing the characters F1+, VW+ and P+ with these characters differently distributed between the constituent members of the combination (*e.g.*, MP6 +++ combined with M33 −−−, M32 +−− combined with M17 −++; M32 +−− combined with M18 −+− and M16 −−+, etc.), to determine whether virulence deficiencies in one strain could be remedied by the presence of a complementary strain. Using doses mixed prior to injection and containing one million organisms of each constituent strain, no evidence of synergistic or antagonistic action was obtained. Where organisms were observable in India ink smears of peritoneal fluid, liver, or spleen derived from moribund animals, their F1 character corresponded with that of the VW+ constituent strain in all cases.

It is therefore apparent that the characters F1+ VW+ P+ permit the expression of high virulence only when they have a closely integrated action within an organism, or within its immediate micro-environment.

Comparative antigenicity of derived strains

Living purine dependent vaccines were tested for ability to evoke F1, V and W antibody response in mice, guinea-pigs and rabbits. At present we have not conclusively demonstrated an antigenic difference between P+ and P− strains and are therefore unable to quote antibody titres relating to this property. Mice progressively gained weight during the period of immunisation. It is interesting to record that, when weighed after dose 8 all immunised mice, except those receiving F1+P+ vaccines, were 5–6 g. heavier than control animals, which then averaged 27·5 g. In an earlier publication (Burrows and Bacon, 1956) we remarked on the health and vigour of mice receiving live plague vaccine. Guinea-pigs also tolerated the vaccines without adverse effects apart from a decrease

in growth rate of groups receiving ++− or −++ vaccines. Rabbits showed some distress for a few days after the initial dose of VW+ vaccines but became tolerant thereafter suggesting that the rabbit may provide a less purine deficient medium than the other species, and that here some multiplication of vaccines may have occurred.

The F1, V and W antibody titres resulting from 12 weeks' immunization of the 3 species with the various vaccines are shown in Table III. It is evident

TABLE III.—*Comparative Antigenicity of Vaccines*

	Live vaccine			Antibody titres of sera produced in: Mouse			Guinea-pig			Rabbit		
	F1	VW	P	F1	V	W	F1	V	W	F1	V	W
M21	+	−	−	32	0	0	64	0	0	8	0	0
M20	+	−	+	32	0	0	64	0	0	8	0	0
M29	+	+	−	32	0	2	32	0	4	32	8	1
M1	+	+	+	32	0	2	32	0	2	32	4	4
M26A	−	+	−	0	2	8	0	0	4	0	16	16
M22A	−	+	+	0	0	16	0	0	2	0	4	4
M28	−	−	−	0	0	0	0	0	0	0	0	0
M27	−	−	+	0	0	0	0	0	0	0	0	0

Sera collected 7 days after the last of 12 weekly doses of vaccines. Antibody titres: the highest of serial 1 : 2 dilutions of serum giving observable specific lines of precipitation when 0·1 ml. of serum dilution was diffused against 0·033 ml. of " standard " antigen preparations in agar diffusion plates of standard pattern, in which the shortest distance between antigen and antibody reservoir perimeters was 0·65 cm.

from this table that the vaccine strains conform to the characters on which they were selected—*e.g.*, no strain selected as being F1− evoked F1 antibody response in any species, whereas all F1+ strains did so.

Considering firstly antibody to F1, it appears that all F1+ strains elicited similar antibody production in the mouse. In the guinea-pig the presence of VW+ in the vaccine somewhat reduced the response to F1. The reverse was the case in the rabbit where the antigenic response to F1 was considerably enhanced in the presence of VW antigens. In no species did the pigmentation character P appear to influence F1 antibody response.

Turning to V antibody production, a notable feature is the very poor response of mice and guinea-pigs to this antigen. No trace of V antibody was detected by our methods in any serum from guinea-pigs and only a small amount found in serum from the group of mice immunised with −+− vaccine. The response of rabbits to V antigen was, however, more vigorous and all VW+ vaccines caused the production of detectable V antibody. The presence of F1+ did not materially affect the response of rabbits to V antigen, but there is a good indication that in the presence of P+ this response is reduced. We do not consider two-fold differences in V and W antibody titres to be significant in view of the poor definition of their lines of precipitation at low concentration.

W antigen produced readily detectable amounts of antibody in all 3 species; the response of guinea-pigs was on the whole inferior to that of mice or of rabbits. In mice but not in guinea-pigs there was a clear indication that the response to W antigen was greatly reduced in the presence of the highly antigenic F1. A similar reduction occurred in rabbits in the presence of F1+P− and also in the presence

of F1—P+. In mice and guinea-pigs there was no clear indication that the P character influenced W antibody production.

Comparative efficiences of vaccines in producing active immunity in mice and guinea-pigs

Groups of 10 mice were challenged, after receiving 8 doses of vaccine, with the virulent strain MP6 +++ or the virulent M23 −++. Similar groups were challenged after 12 doses. Since the responses to the two types of challenge were similar after 8 and 12 doses of vaccine we have combined the results to form Table IV.

TABLE IV.—*Active Immunogencitiy of Vaccines in Mice*

	Live vaccine			Challenge organism: MP6 +++			Challenge organism: M23 −++		
	F1	VW	P	M	T	A.P.I.	M	T	A.P.I.
M21	+	−	−	10	4·5	45	45	9·4	21
M20	+	−	+	10	14	140	45	9·7	22
M29	+	+	−	5	17	170	20	9·8	49
M1	+	+	+	0	>21	>210	25	8·2	33
M26A	−	+	−	35	8	23	10	11	110
M22A	−	+	+	5	7	140	5	6·0	120
M28	−	−	−	95	4·0	4·2	40	5·5	14
M27	−	−	+	95	4·4	4·6	40	8·0	20
Nil		..		100	2·7	2·7	100	4·0	4·0

Groups of 20 mice (combined results of 2 experiments using groups of 10 mice which had received 8 and 12 doses of vaccines at 7-day intervals). Challenged 7 days after last vaccine dose with 2000 MP6 or M23 i.p. in 0·1 ml. of buffer.

M = mortality per cent.

T = mean time of death in days.

A.P.I. = active protection index = $\frac{T}{M} \times 100$.

Considering protection against challenge with MP6 the striking feature is the complete failure of strains lacking both F1 and VW to confer more than the slightest protection despite the lengthy period of immunisation. Vaccines having the character F1+ or VW+, and particularly those having both, are highly protective. Comparing the active protection index (A.P.I.) of a vaccine which is " + " for a particular character with the A.P.I. of the vaccine which is " − " for the same character it is seen that, in the four possible pairs, vaccines which are F1 + give a higher A.P.I. than the coresponding F1— vaccine (M21 v. M28; M20 v. M.27; M29 v. M26A and M1 v. M22A). Similarly all VW+ vaccines are more protective than their VW— counterparts. Although having little protective action by itself, the character P+ contributes materially to the immunogenicity of F1+ or VW+ vaccines, since again in all 4 comparisons the P+ vaccines have higher indices than have P—.

Against challenge with M23, strains lacking both F1 and VW have moderate protective action in contrast to their failure to protect against challenge with MP6. Highest protection is afforded by VW+ vaccines. particularly those lacking F1. Comparing active protection indices as above, all 4 VW+ vaccines are superior to their VW— counterparts, 2 F1+ vaccines are higher and 2 lower than the corresponding F1— vaccines, and 3 P+ are higher and one lower than

the corresponding P— although for this character the differences are very small, and may be meaningless.

Groups of 5 guinea-pigs were challlenged after receiving 8 vaccine doses. The results are shown in Table V. The general picture following challenge with MP6

TABLE V.—*Active Immunogencity of Vaccines in Guinea-pigs*

Live vaccine				Challenge organism: MP6 +++			Challenge organism: M23 −++		
	F1	VW	P	M	T	A.P.I.	M	T	A.P.I.
M21	+	−	−	0	>21	>210	20	9	45
M20	+	−	+	0	>21	>210	60	13	22
M29	+	+	−	0	>21	>210	0	>21	>210
M1	+	+	+	20	17	85	20	18	90
M26A	−	+	−	20	6	30	20	17	85
M22A	−	+	+	60	8	13	40	12	30
M28	−	−	−	20	12	60	0	>21	>210
M29	−	−	+	40	8	20	80	12	15
Nil		..		100	5	5	60	5	8·3

Groups of 5 guinea-pigs.
Challenged 7 days after 8th vaccine dose with 1 million MP6 or M23 subcutaneously.
M, T, and A.P.I., as in Table IV.

was that all vaccines conferrred some immunity but there was a clear superiority of F1+ over F1— vaccines and a superiority of P— over P+ vaccines. VW+ evidently detracts from the protective value of vaccines for guinea-pigs in marked contrast to its behaviour in mice. Another notable feature is the high efficiency shown by ——— vaccine, again in contrast to its effect in mice. The high protective action of F1+ vaccines suggests that F1 antigen, as it occurs in the living organism, is of considerable importance for immunity in guinea-pigs in contrast to previous findings using killed organisms or extracted antigen.

Our results following challenge with −++ M23 may not be very meaningful in view of the relatively low virulence of this strain for the guinea-pig, with the consequent failure to produce 100 per cent mortality in control animals in this test. Nevertheless the failure of the majority of vaccines to confer solid immunity against the relatively low challenge employed serves to emphasise the difficulty of immunising these animals against infection with this strain. This difficulty presumably results from the indifference of M23 to F1 antibody and from the inability of the guinea-pig to respond effectively to V and W antigens. As with challenge with MP6 there was a clear superiority of P— over P+ vaccines.

Passive protection of mice : rabbit antisera

The results of attempts to passively protect mice against challenge with MP6 and with M23, using antisera produced against the different vaccines in rabbits, are recorded in Table VI. High protection against MP6 challenge was confined to sera derived from rabbits immunised with F1+VW+ vaccines. Such vaccines evoked the highest F1 antibody response (Table II) so that sera produced against them may have owed their greater protective ability either to their high F1 antibody or to the combined action of F1 and VW antibodies. Sera produced against vaccines lacking both F1 and VW were virtually devoid of protective action. A comparison of the passive protection indices (P.P.I's) of corresponding

TABLE VI.—*Passive Protection of Mice with Rabbit Antisera*

Rabbit anti-serum	Immunization of rabbits: 8 weeks challenge: MP6+++ M	T	P.P.I.	8 weeks challenge: M23−++ M	T	P.P.I.	12 weeks challenge: MP6+++ M	T	P.P.I.	12 weeks challenge: M23−++ M	T	P.P.I.
M21 +−−	100	4·4	4·4	100	3·2	3·2	100	5·8	5·8	100	3·2	3·2
M20 +−+	80	5·0	6·3	100	3·1	3·1	100	5·1	5·1	100	3·3	3·3
M29 ++−	40	14·0	35	90	5·6	6·2	20	12·5	63	90	9·1	10
M1 +++	30	13·7	46	90	6·1	6·8	30	10·3	34	90	10·1	11
M26A−+−	100	3·8	3·8	100	5·0	5·0	90	9·7	10	90	8·8	9·8
M22A−++	100	5·1	5·1	90	9·3	10	100	6·1	6·1	100	7·8	7·8
M28 −−−	100	2·6	2·6	100	3·0	3·0	100	3·1	3·1	100	3·6	3·6
M27 −−+	100	2·5	2·5	100	3·3	3·3	100	3·0	3·0	100	3·3	3·3
Normal	100	2·0	2·0	100	2·9	2·9	100	3·0	3·0	100	3·1	3·1

Groups of 10 mice.
Serum 0·2 ml. intraperitoneally followed 1 hr. later by 2000 MP6 or M23 i.p. in 0·1 ml. buffer.
M = mortality per cent; T = mean time of death in days; P.P.I. = passive protection index $= \frac{T}{M} \times 100$.

pairs of sera (as was done to compare A.P.I's) shows that of the 8 pairs in the 2 tests, all 8 F1+ antisera have higher indices than their F1− counterparts, all 8 VW+ are higher than VW−, while 3 P+ are higher and 5 lower than the corresponding P− antisera, when used to protect against MP6 challenge.

Against challenge with M23, demonstrable protection was confined to antisera produced against VW+ vaccines. Similar comparison of P.P.I's to that above can be scored thus: F1+ 4 higher, 3 lower; VW+ 8 higher, 0 lower; P+ 5 higher and 3 lower. (Mice are however readily protected against M23 challenge by hyperimmune rabbit sera having V antibody titres of 32 or higher.)

Passive protection of mice: guinea-pig antisera

Protective action against MP6 challenge was clearly confined to antisera produced with F1+ vaccines (Table VII). Although these antisera had equally

TABLE VII.—*Passive Protection of Mice with Guinea-pig Antisera*

Guinea-pig anti-serum	Immunization of guinea-pigs: 8 weeks challenge: MP6+++ M	T	P.P.I.	8 weeks challenge: M23−++ M	T	P.P.I.	12 weeks challenge: MP6+++ M	T	P.P.I.	12 weeks challenge: M23−++ M	T	P.P.I.
M21 +−−	100	9·2	9·2	100	3·1	3·1	90	10·7	12	100	3·0	3·0
M20 +−+	70	11·0	16	100	3·0	3·0	90	13·5	15	100	3·1	3·1
M29 ++−	70	11·0	16	100	3·4	3·4	90	9·9	11	100	3·0	3·0
M1 +++	90	9·8	9·8	100	3·5	3·5	90	8·0	8·8	100	3·0	3·0
M26A−+−	100	4·3	4·3	100	8·0	8·0	100	2·1	2·1	100	3·0	3·0
M22A−++	100	2·7	2·7	100	3·2	3·2	100	2·3	2·3	100	2·9	2·9
M28 −−−	100	2·5	2·5	100	3·1	3·1	100	2·5	2·5	100	3·7	3·7
M27 −−+	100	2·8	2·8	100	3·0	3·0	100	2·4	2·4	100	2·9	2·9
Normal	100	2·6	2·6	100	3·0	3·0	100	2·1	2·1	100	2·7	2·7

Procedure the same as described in Table VI.

high, or higher, F1 antibody titres than corresponding antisera produced in rabbits, their passive protective abilities did not approach those shown by sera produced in rabbits using F1+VW+ vaccines. It is probable therefore that the high passive protective abilities of the latter resulted from the combined action of F1 and VW antibodies. Comparison of P.P.I's as above gives the following: F1+ 8 higher, 0 lower; VW+ 2 higher, 6 lower; P+ 4 higher and 4 lower. Sera produced in guinea-pigs were devoid of protective action against M23 challenge.

Comparative immunogenicity of purine dependent and independent strains

We thought it possible that a comparison of the immunogenicity of corresponding purine dependent (Pu−) and independent (Pu+) live vaccines, over a brief period of immunisation, would provide information on the extent of multiplication of Pu+ vaccines *in vivo*. Groups of mice were injected with one million live organisms of purine dependent and independent vaccine strains and challenged one week later with MP6. Similar groups received two doses of one million organisms at one week interval and were similarly challenged. Groups of guinea-pigs were compared in like fashion, after receiving one or two doses of one million organisms. The active protection indices calculated from these experiments are shown in Table VIII.

TABLE VIII.—*Active Protection Indices Given by Purine Dependent (Pu−) and Independent (Pu+) Vaccines in Mice and Guinea-pigs*

Virulence characters of vaccines			Mice. Immunization				Guinea-pigs. Immunization			
			One dose		Two doses		One dose		Two doses	
F1	VW	P	Pu−	Pu+	Pu−	Pu+	Pu−	Pu+	Pu−	Pu+
+	−	−	3·6	4·2	5·9	11	8·0	80	>210	>210
+	−	+	4·1	6·6	4·7	>210	9·6	>210	65	>210
+	+	−	9·6	>210	30	>210	23	>210	>210	>210
+	+	+	28	*	40	*	11	*	>210	*
−	+	−	6·0	11	5·3	9·2	9·6	35	28	>210
−	+	+	6·1	*	17	*	7·4	*	22	*
−	−	−	3·2	3·9	3·0	3·7	7·5	12	14	>210
−	−	+	3·4	3·9	2·9	2·9	10	12	29	>210
Nil	.	.	3·8	3·8	3·0	3·0	6·5	6·5	7·4	7·4

Groups of 10 mice or 5 guinea-pigs.
Active protection index calculated as in Table IV.
* Not tested since strain of high virulence at dosages used.
Immunization: 1 or 2 doses of 10^8 live organisms at 7-day interval, challenged 7 days after last dose.
Challenge: Mice 2000 MP6 i.p.
Guinea-pigs 10^8 MP6 s.c.

From single doses of vaccines in mice the respective active protection indices, resulting from corresponding purine dependent and independent vaccines, did not differ greatly, excepting for those from ++− strains. In this case that from the Pu+ strain considerably exceeded that from the Pu−. Assuming that the resulting A.P.I. is directly related to the mass of protective antigen(s) available for antibody production and that Pu− strains are not able to multiply *in vivo* we must conclude that, of the Pu+ vaccines, only that which was ++− multiplied significantly. It is known that ++−Pu+ strains are capable of limited growth

in normal mice and that they can achieve the high populations characteristic of fully virulent strains in the presence of low levels of injected iron (Jackson and Burrows, 1956).

On comparing the indices resulting from two doses of vaccines great difference between those from corresponding Pu— and Pu+ vaccines is seen not only with ++— strains but also with +—+ strains. It is difficult to explain the higher index of +—+Pu+ on the basis of multiplication of this vaccine, since this would involve greater multiplication of the second dose than of the primary dose, and it is equally difficult to offer an explanation based on the " boosting " effect of the second dose since the second dose of the Pu— vaccine did not greatly increase the A.P.I.

Corresponding Pu+ and Pu— vaccines differed considerably in the protection they afforded to guinea-pigs from a single dose. The differences were particularly marked in those vaccines bearing the highly immunogenic character F1+, indicating a considerable multiplication of such Pu+ vaccines in this species. Second doses of all F1+Pu— vaccines in the guinea-pig greatly enhance the immunity derived from a single dose, in contrast to their behaviour in mice. This could be explained as a boosting effect. The high immunogenicity of two doses of F1—Pu+ vaccines however cannot be so explained since the corresponding F1—Pu— vaccines do not show a very evident boosting action. These observations may indicate that strains capable of metabolism in the guinea-pig (*i.e.*, Pu+ strains) elaborate a protective antigen not formed during growth *in vitro*, or by Pu— strains *in vivo*, and which requires a boosting dose to provide highly protective levels of antibody.

It is interesting to note that the vaccine strains here shown to be pre-eminent for the active immunisation of both mice and guinea-pigs have the virulence characters of the strains EV76 (++—) and Tjiwidej (+—+), both of which, on the basis of their efficiency for laboratory animals, were selected for use as vaccines in man.

DISCUSSION

Our assessment of the comparative virulence of genetically related strains leaves no doubt that full virulence for mice and guinea-pigs is intimately associated with the character VW+. Of the 3 virulence characters investigated here namely F1+, VW+ and P+, loss only of the VW+ character leaving F1+ and P+ intact, results in complete loss of virulence for both the above species. Loss of P+ alone renders a strain avirulent for the guinea-pig and reduces virulence for mice ; loss of F1+ alone does not affect virulence for mice but reduces virulence for the guinea-pig. Strains dependent on an exogenous supply of purines for growth are avirulent for both mice and guinea-pigs, regardless of their complement of the above virulence determinants, as a result of nutritional limitation *in vivo*. There are indications however that such strains may not be so strictly limited in the rabbit.

It is obvious that no simple explanation can be offered to account for the different antibody responses of mice, guinea-pigs and rabbits which received the same batches of the different vaccines. The 3 species have the common ability to respond vigorously to the presence of F1 antigen when this is presented in a living vaccine. Similarly they all respond, though less vigorously, to the presence of W antigen. In contrast V antigen evokes readily demonstrable amounts of

antibody only in the rabbit. In guinea-pigs it appears to be non-antigenic on the evidence of serum analyses by the diffusion method, the results of active protection tests and the inability of guinea-pig antisera to confer passive protection on mice challenged with M23. Although mice respond very poorly to the presence of V antigen, as judged by humoral antibody titres, vaccines containing VW+ confer considerably higher active immunity to mice against challenge with MP6 or M23 than do corresponding VW− vaccines. This higher immunity could be due to W antibody alone. Two pieces of evidence contradict this interpretation however; firstly, guinea-pig antiserum containing measurable amounts of W antibody are non-protective against M23 challenge in mice, and secondly the passive protection afforded to mice by rabbit antisera, against challenge with M23, seems not to be related directly to the W antibody content of these antisera. The definite solution of problems of this kind must await the production of purified V and W antigens.

V antigen is not the only product of *Past. pestis* showing differing antigenicity in the 3 species. When examined by the agar diffusion technique antisera produced in guinea-pigs by −−+ and −−− vaccines can be shown to have two antibodies not possessed by antisera produced in rabbits or in mice; on the other hand antisera produced in the latter 2 species have a major antibody which is apparently absent from corresponding antisera produced in guinea-pigs.

This comparative study lends full support to the view that F1 antigen is of great importance in conferring immunity to mice against experimental infection with virulent F1+ strains. It has, in addition, shown that VW antigens are not less important for this purpose. Maximum protection is achieved by the use of vaccines containing both F1 and VW. Against the virulent F1− strain M23, F1 antigen has no protective action. High protection against this strain is only conferred by VW+ vaccines, which appear to have their protective action reduced if accompanied by the character F1+. Although we have not encountered F1−VW+P+ strains analogous to strain M23 in the culture collections we have examined, such strains are readily selected in the laboratory. In mice immunized with F1+VW− vaccines and challenged with F1+VW+ virulent strains a strong selective pressure exists in favour of any F1−VW+ organisms present in the challenge dose, or arising as a result of its multiplication, and it is not uncommon for the terminal population in mice which subsequently die to consist largely or exclusively of F1− VW+ organisms.

This study lends no support to the view that F1 antigen is of minor importance for the protection or guinea-pigs against F1+ virulent infection. On the contrary we would conclude that, as it occurs in the living organism, this antigen is of primary importance for this purpose. An antigen other than F1, VW or P, present in −−− vaccines (presumably " residue antigen ") contributes materially to the protection of guinea-pigs against F1+ challenge in contrast to its inefficiency in mice. Again in complete contrast to its effects in mice the character VW+ detracts from the protective activity in vaccines in guinea-pigs as also does the character P+. Although we refer to the ability of strains to form pigmented colonies as being a positive attribute (P+) this ability may be attributable to the absence of a product possessed by strains we designate as P−. This product may be antigenic and play an important role in immunity. Therefore instead of saying that P+ detracts from immunogenicity it may be more correct to say that P− contributes to immunogenicity in guinea-pigs.

The interpretation of the relative importance of the virulence characters F1, VW and P in the production of immunity is complicated by the apparent effects of one character on the antigenicity of another. As examples, the antigenicity of W antigen in mice was reduced if the vaccine was F1+ (or, conversely, was increased if the vaccine was F1−). This apparent influence of F1 on the antigenicity of W did not operate in guinea-pigs, which received the same batches of vaccines. Similarly in the rabbit P+ apparently reduced the antigenic response to V antigen. Explanation of these observations must be speculative at this stage. We incline to the view that the living organism produces a complex containing F1 and possibly VW and other antigens, which is differently fragmented in different animal species to give smaller components the antigenicity of which is determined perhaps by the size of the components and the nature of the antigens exposed. We already have evidence that material with F1 specificity can exist in the intact F1+ organism as a non-diffusable, and therefore presumably very large molecular size, complex from which diffusable F1 can be liberated by ultrasonic treatment, autolysis, or acetone drying. Our findings concerning the immunogenicity of living F1+ organisms for guinea-pigs could be explained by the high antigenicity of the F1 complex as opposed to a low antigenicity of smaller, diffusable F1 antigen, in this species.

At this point it is appropriate to refer to 2 interesting strains M24 and M30, which were respectively isolated from the purine dependent strains M1 and M20 when we were selecting F1− mutants. These form unstable suspensions when grown in broth at 37°, are not enveloped when grown *in vitro* at this temperature or when grown *in vivo*, and are not agglutinable by specific F1 antisera. On these criteria M24 and M30 would be regarded as F1− strains. However, by the diffusion technique of antigen analysis these 2 strains can be shown to produce similar amounts of material having F1 specificity to those produced by F1+ strains. It is hoped that the current study of M24 and M30 together with a comparable, but purine independent, strain " Bryan " (which was recently isolated from a human plague fatality) may shed some light on the states of F1 antigen in nature.

Our comparison of the protection afforded by short term immunisation with corresponding Pu+ and Pu− vaccines strongly suggests that the former may elaborate a protective antigen (or antigens) when metabolising in the guinea-pig which they fail to produce effectively by growth *in vitro*. If this could be proved by other methods the high efficiency of living vaccines in conferring active protection on guinea-pigs would have a simple explanation.

SUMMARY

A comparative study was made of the virulence and immunogenicity of genetically related strains of *Pasteurella pestis* having all permutations of the virulence determinants F1+, VW+ and P+. Two corresponding series of strains were selected, one of which was dependent (Pu−) and the other independent (Pu+) on an exogenous supply of purines for growth. Virulence studies supported previous conclusions. The studies on immunity to experimental plague revealed a complex interaction of factors which prevents a brief summary being made ; many statements would require qualification as to the animal species, the method of immunization and the nature of the challenge employed. Mice, guinea-pigs and rabbits differed in their antibody response to living vaccines and, in one or other of these

species, the antigenic response to one determinant appeared to be modified by the presence of a second determinant in the vaccine.

Mice were best protected against F1+ virulent challenge by F1+VW+P+ vaccines; F1+ VW+ vaccines were greatly superior to F1+VW− vaccines, while F1−VW− vaccines whether P+ or P− were non-protective. In guinea-pigs F1+ vaccines were more highly protective than F1−, and P− more protective than P+. In this species the determinant VW+ detracted from the active immunising ability of vaccines. Comparison of the immunogenicity of Pu+ and Pu− vaccines in guinea-pigs indicated that the former produce a protective antigen *in vivo*. F1 and W antigens gave detectable humoral antibody in all three species, whereas V antigen gave good response in rabbits, very poor response in mice and appeared to be non-antigenic in guinea-pigs.

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THE HISTOCHEMISTRY OF INDOXYLESTERASE OF RAT KIDNEY WITH SPECIAL REFERENCE TO ITS CATHEPSIN-LIKE ACTIVITY

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CHANGES affecting the hydrolytic enzymes of the nephron in various metabolic disorders have been studied histochemically by a number of authors (Wachstein, 1955; Wachstein and Meisel, 1957; Macpherson and Pearse, 1957; Pearse and Macpherson, 1958). The characteristics of the renal carboxylic acid esterases in particular, however, have not been properly established. Using various α-naphthyl substrates and various inhibitors, Gomori (1955) obtained evidence of an eserine-resistant carboxylic acid esterase in human kidney. This enzyme was partially inhibited by organophosphorus compounds and showed some of the features of a lipase. From the work of Aldridge (1954) it became evident that the use of a variety of substrates was less successful in differentiating esterase activities than the use of various activators and inhibitors. Although a number of naphthyl and indoxyl substrates are available for histochemical use, distinction between the different types of esterase is probably better made by the use of a single substrate. Advantage can be taken, therefore, of the precise intracellular localization offered by the halogen-substituted indoxyls (Holt, 1956).

Aldridge (1954) separated the carboxylic acid esterases into two types (A and B) by their sensitivity towards organophosphorus inhibitors. In the present study, advantage was taken of the fact that E600 (diethyl-*p*-nitrophenylphosphate) inhibits irreversibly the B-type esterases only, allowing the properties of the A-type to be investigated. Histochemical studies (Pearse, 1956; Pepler and Pearse, 1957*a* and 1957*b*; Pearse and Macpherson, 1958) of the indoxyl esterases of hypophysis, brain, thyroid and kidney suggested that part of the observed esterolytic activity was due to a proteolytic enzyme, since several of these enzymes are known (cf. Neurath and Schwert, 1950) to be capable of splitting esters as well as peptides. In the present paper it will be shown that in fact a cathepsin-like enzyme is partially responsible for the histochemical demonstration of A-type esterase by the indoxyl method.

MATERIALS AND METHODS

Stock hooded male rats, weighing 200–350 g. were used throughout. The animals were killed by decapitation and the kidneys, part of the liver, and the pancreas were removed and fixed for approximately 24 hr. in cold (4°) formalin-calcium. Sections were cut on a freezing microtome at 7·5 μ, and after brief washing in distilled water were used for esterase studies. For the demonstration of leucine aminopeptidase, fresh frozen sections, cut at 8–15 μ on a remotely-controlled cold microtome (cryostat), were used.

The esterase method was performed according to Holt and Withers (1952) using *o*-acetyl-5-bromoindoxyl as a substrate. Free-floating sections were incubated for 2 hr. at 37°. In the pH studies *o*-butyryl indoxyl was tested in addition to the acetate.

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In order to compare the esterase activity with that shown by a substrate specific intracellular proteolytic enzyme, we used the method of Nachlas, Crawford and Seligman (1957) for the demonstration of leucine aminopeptidase (pH 6·5) with L-leucyl-β-naphthylamide hydrochloride as a substrate.

The B-type esterases were eliminated by incubation of the sections in 10^{-5} M E600 (diethyl-*p*-nitrophenylphosphate) in buffer for 1 hr. at 37°. This treatment resulted in an approximately two-thirds loss of esterase activity, except in certain sites referred to below. In control studies, 10^{-3} M Mipafox (NN′-di-*iso*-propylphosphorodiamidic fluoride) was shown to have the same effect as 10^{-5} M E600. After this treatment the sections, containing only E600-resistant enzyme, were transferred to solutions of various activators and inhibitors (see Table) in buffer pH 5·3 and incubated for 1 hr. Thereafter the tissues were immersed in the substrate mixture at pH 5·3, containing the appropriate activator or inhibitor.

TABLE.—*Inhibition and Activation of E600-resistant Rat Kidney Esterase*

Substance	Concentration	Proximal tubules	Distal tubules*	Epithelium of the papilla
Ascorbic acid	2×10^{-3} M	+1	+1	+1
Cysteine	1×10^{-3} M	+1	+1	0
Glutathione	1×10^{-3} M	+1	+1	0
β-phenylpropionic acid	1×10^{-2} M	−2	−2	−2
D-L phenylalanine	1×10^{-3} M	−1	−1	−1
Sodium citrate	1×10^{-2} M	−1	−1	−1
Sodium fluoride	1×10^{-3} M	−1	−1	0
Iodoacetic acid	5×10^{-3} M	−1	−	−2
Potassium cyanide	2×10^{-3} M	−1	−1	−1
Magnesium chloride	5×10^{-2} M	0	0	0
Cupric sulphate†	1×10^{-3} M	−1	−1	−1
Lead nitrate†	1×10^{-3} M	−2	−2	−2
2 : 4 dinitro-1-fluorobenzene	1×10^{-3} M	0	0	0
Tween 60	10^{-3} g./100 ml.	+2	+2	+1
Tween 80	10^{-3} g./100 ml.	+2	+2	+1
Trypsin cryst.‡	1/2 sat. soln.	+2	+2	+2
Phenylmercuric chloride	1×10^{-4} M	+1	+1	0

* Strongly esterase positive intercalated cells.
† Only pre-incubation used, substance not added to substrate.
‡ Pre-incubation in trypsin pH 7·8 at 25° for ½ hr.

Arbitrary grading of enzyme activity—estimated visually, using the following figures: −, inhibition; +, activation; 1 = 25 per cent; 2 = 50 per cent—compared with control sections showing zero activity.

The inhibitor and activator studies were also carried out with control sections.

To ensure the absence of bacteria or fungi, which possess enzymes capable of hydrolyzing indoxyl esters, heated or freshly prepared Veronal-HCl buffer was used throughout.

In vitro studies were performed with crystalline pancreatic chymotrypsin (Bios Laboratories Inc.), trypsin and pepsin (Nutritional Biochemicals Corporation), and carboxypeptidase (L. Light & Co.), obtained commercially and used without further purification. In addition, a crude preparation of papain was tested. Different enzyme concentrations were prepared in ice-cold veronal-HCl buffer (5×10^{-2} M) at the optimal pH of the enzyme under investigation. In the case of carboxypeptidase, the ionic strength of the solutions was maintained with NaCl at 0·2. The concentration of the substrate (*o*-acetyl indoxyl) was restricted by the low solubility of this compound in water to 6×10^{-3} (*cf.* Underhay, 1957). Incubation was carried out at 25° or at 37°. One ml. of the reacting mixture was withdrawn every 15 min. and was vigorously shaken with chloroform for 10 min. The indigotin was extracted after centrifugation. The chloroform was allowed to evaporate in the dark, the dye was redissolved in NN′-dimethylformamide and measured immediately in a spectrophotometer. The enzyme concentration was determined in terms of nitrogen per ml. by the micro-Kjeldahl method.

RESULTS

Histochemical Methods

pH-series

It was found that in rat kidney, esterase activity exhibits two pH-optima, one from pH 4·6 to 6·0 and a second from pH 7·0 to 7·5. This finding suggests the existence of at least two enzymes capable of splitting *o*-acetyl-5-bromoindoxyl. Using *o*-butyryl indoxyl, only one pH-optimum (7·4 to 7·7) was detected. The cleavage of *o*-acetyl-5-bromoindoxyl occurred, in a moderate degree, at both pH 2·6 and pH 9·6.

Distribution of the organophosphorus-resistant esterase

Treatment with E600 caused approximately a two-thirds loss of the total indoxyl esterase activity. This effect was easily reproducible and irreversible. The inhibition, however, did not affect all the tissue elements to the same degree. The proximal tubules, as a whole, suffered a loss of esterase activity. The large intracytoplasmatic droplets, which are normally found in older male rats, showed a strongly resistant esterase (Fig. 3). The descending and ascending limbs were almost devoid of activity. No enzyme could be demonstrated in the thin segment and in the collecting ducts of the outer medulla. Interstitial macrophages and certain cells of the distal tubules and collecting ducts of the cortex contain an esterase which shows a striking resistance to E600 (Fig. 1). These tubular cells are intercalated in the rather flat epithelium of the corresponding ducts. They are usually larger than the esterase-free neighbouring cells and protrude into the lumen of the tubule. The esterase is concentrated in the perinuclear rim and the horns of the crescent so formed point away from the lumen. Occasionally, especially in older animals, these cells are desquamated into the lumen. At present the function of these cells of the distal duct is not clear, but it is suggested that they may be of significance in delayed, distal protein resorption (Mayersbach and Pearse, 1956). They are not identical with intercalated cells of the medullary ducts referred to by Oliver *et al.* (1957).

An organophosphorus-resistant esterase was also present in the pelvic epithelium and in cells of the collecting ducts near the tip of the papilla.

The same resistant enzyme activity could be demonstrated in the Kupffer cells of the liver and to a lesser extent in the liver epithelia surrounding the bile canaliculi. In contrast to the islets of Langerhans and the pancreatic duct epithelium, the zymogen granules of the pancreatic acini exhibited only a faint E600-resistant esterase. This is in perfect agreement with the well known inhibition of most of the active pancreatic enzymes by organophosphorus compounds (Jansen, Nutting, Jang and Balls, 1949).

Properties of the organophosphorus-resistant esterase

The pH-optimum of the E600-resistant esterase is pH 5·0 to pH 5·5, at pH 7·0 activity is markedly decreased. The table summarizes the effect of various activators and inhibitors on the E600-resistant esterase activity of rat kidney at

pH 5·3. The esterase of the Kupffer cells, and of the macrophages of the omentum, behaves in a similar manner.

The properties of the organophosphorus-resistant esterase closely resemble those of an intracellular proteolytic enzyme, *i.e.*, a cathepsin. The activation by trypsin (see Fig. 2 and 4) is a common feature of most of the pancreatic proteases (Neurath and Schwert, 1950). This process is presumed to involve the opening of single peptide bonds in the inactive precursor (Neurath and Dixon, 1957). Trypsin was observed to activate an E600-resistant indoxyl esterase in sections of rat pancreas. The precursors of the pancreatic proteolytic enzymes are not sensitive towards organophosphorus inhibitors. The esterase activity of the proteolytic enzymes is referred to below.

The activation of pancreatic carboxypeptidase by detergents has been described by Gorini and Labouesse-Mercouroff (1954). Histochemically, this effect was most marked, but we failed to increase esterase activity by raising the ionic strength of the incubating mixture to 0.62, a procedure referred to by the same authors.

Cysteine, glutathione, and in certain cases ascorbic acid, activate various cathepsins (Smith, 1951). This action of reducing agents is presumably due to opening of the S–S bonds of the enzyme molecule. The participation of SH-groups in the enzymatic process is suggested also by the inhibitory effect of iodoacetic acid and heavy metal ions.

Inhibition by iodoacetate, D-amino-acids and cyanide, is a property of the carboxypeptidases (Elkins-Kaufmann and Neurath, 1948) and these, as metalloenzymes, are also inhibited by citrate (Vallee, 1955). Histochemical attempts to produce inhibition by chelating agents have been unsuccessful, because of interference with the oxidizing catalyst (0·2 M ferricyanide and 0·2 M ferrocyanide) in the substrate mixture. Mg-ions in high concentration were also without effect. Another metalloenzyme, leucine aminopeptidase, showed an entirely different pattern in the rat kidney when compared with the E600-resistant esterase. As pointed out by Nachlas, Crawford and Seligman, only the tubules of the inner cortex are strongly stained. Leucine aminopeptidase survived only a short formalin fixation, but was resistant to organophosphorus inhibitors. Highly purified leucine aminopeptidase from swine kidney shows very little esterase activity (Smith and Spackman, 1955). The ester substrates of carboxypeptidase do not require a hydrogen atom or a peptide nitrogen at the sensitive bond (Snoke, Schwert and Neurath, 1948), but the presence of a free carboxyl group seems to be essential for the action of this enzyme. Therefore, *o*-acetylindoxyl and *o*-acetyl-5-bromoindoxyl are not attacked by pancreatic carboxypeptidase.

The effect of phenylmercuric ions in low concentration suggests the presence of a " C-esterase ", recently described in hog kidney extract by Bergmann, Segal and Rimon (1957).

Inhibition by β-phenylpropionic acid suggests a chymotrypsin-like activity. This inhibition is competitive in nature and it could be demonstrated in the tissues only in relatively high concentrations. Chymotrypsin is stable to the action of formaldehyde and apparently requires no amino groups for its enzymatic activity (Neurath and Schwert, 1950).

The insensitivity of the E600-resistant enzyme activity to heat (60° for 30 min.) parallels that of a salicyl butyrate-splitting " Esterase II " of rat pancreas, described by Myers, Schotte, Boer and Borsje-Bakker (1955).

In vitro *Experiments*

Of the crystalline proteases used, only chymotrypsin was found to hydrolyze *o*-acetylindoxyl at a measureable rate. The results obtained with two different concentrations of chymotrypsin are presented in Fig. 5. The liberated indoxyl, measured as indigotin, its oxidation product, is plotted against the time of the

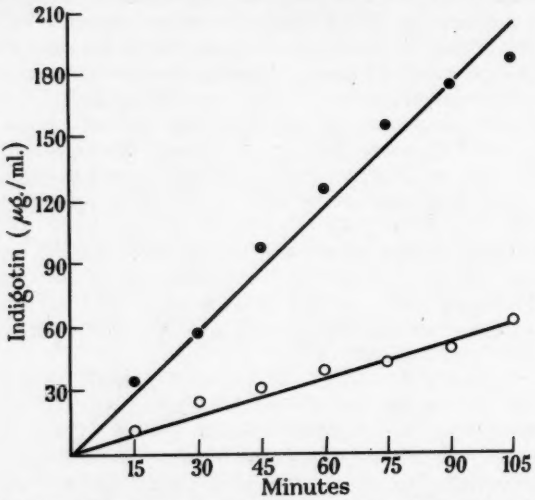


FIG. 5.—Indigotin in micrograms produced by the hydrolysis of *o*-acetyl indoxyl by chymotrypsin at two different concentrations in 5×10^{-2} M veronal-HCl buffer at pH 7·8 and 25°. The amount of chymotrypsin nitrogen in these systems is shown by ○ = 0·25 mg. N/ml., ● = 0·44 mg. N/ml. Indigotin measured spectrophotometrically at 590 mμ in N,N′-dimethylformamide.

reaction. The relationship of dye produced to enzyme concentration is a linear one and the reaction is probably of zero order. Our experimental results are not sufficiently accurate to determine the kinetics, however, on account of the low solubility of the substrate in water.

A feeble reaction was obtained with Mg^{++}-activated crystalline trypsin at a comparatively high enzyme concentration. These results were not reliable, however, because bacterial contamination of the reacting medium could not be excluded. A rapid hydrolysis of *o*-acetylindoxyl was achieved with crude papain.

EXPLANATION OF PLATE:

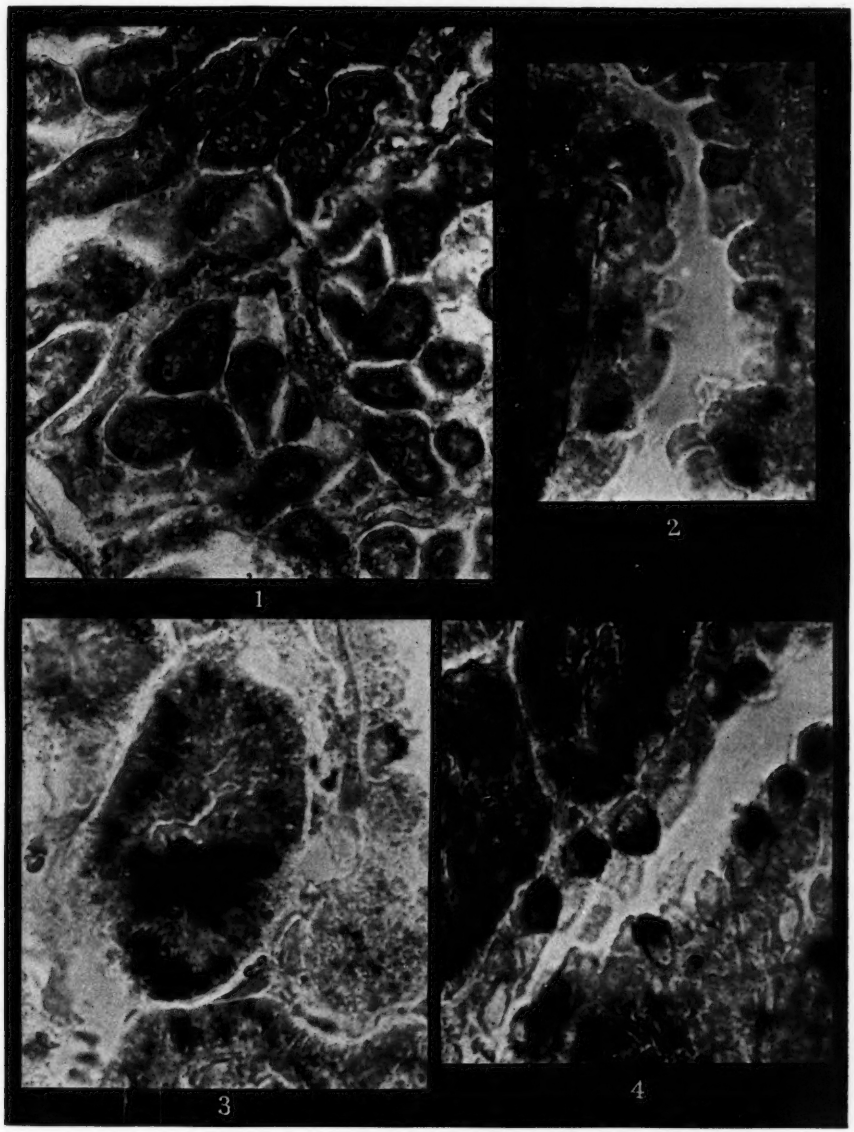
Preparations in Fig. 1–4 are of 7·5 μ cold formol-calcium fixed frozen sections of rat kidney.

FIG. 1.—Part of the outer cortex. The distal tubules and the first part of the collecting tubules show indoxyl esterase activity restricted to intercalated cells (no inhibitor used). × 215.

FIG. 2.—Distal tubule shows E600-resistant indoxyl esterase in intercalated cells (control). × 850.

FIG. 3.—Kidney of old male rat shows E600-resistant indoxyl esterase concentrated in large droplets in the cells of a proximal tubule. × 850.

FIG. 4.—Same kidney as Fig. 3. Activation of E600-resistant indoxyl esterase by preincubation with trypsin (½ hour, 25°, pH 7·8). × 850.



Hess and Pearse.

DISCUSSION

In the present paper we describe an organophosphorus-resistant indoxylesterase in the proximal tubules, in certain intercalated cells of the distal tubules and in the pelvic epithelium of rat kidney, which has the characteristics of a cathepsin. A similar enzyme was found in macrophages, in Kupffer cells of the liver, in the islets of Langerhans and in pancreatic duct epithelium. It is probably identical with the non-specific esterase described in pituitary mucoid cells by Pearse (1956), in rat thyroid, rat brain and human argentaffin cell tumours by Pepler and Pearse (1957*b* and 1957*a*) and Pearse and Pepler (1957).

This enzyme system belongs to the A-type esterases ("Aromesterases") investigated by Aldridge, or to the "Organophosphate-resistant Esterase" of Hobbiger (1957). The differentiation of the esterases is possible only by using specific inhibitors, because the overlap in substrate specificity of these enzymes is considerable (Aldridge). Myers *et al.* (1955) suggested that E600-resistant and heat-resistant esterase of rat pancreas might be related to a proteolytic enzyme, differing from chymotrypsin or trypsin. Histochemically, indoxyl esterase has been shown to have similar properties.

The biochemical evidence for the esterase activity of pancreatic proteolytic enzymes has been reviewed by Neurath and Schwert (1950). This activity, investigated mostly with synthetic amino-acid ester substrates, is exhibited also by cathepsins. These intracellular proteolytic enzymes act on proteins or on synthetic substrates for well characterized proteases and peptidases, but are optimally active at an acid pH. A new classification of the animal cathepsins, based on substrate specificities, was proposed by Tallan, Jones and Fruton (1952). Their "Cathepsin A" is comparable to pancreatic pepsin, "Cathepsin B" to trypsin and "Cathepsin C" to chymotrypsin. In addition, leucine aminopeptidase (and other aminopeptidases), a carboxypeptidase and a tripeptidase were distinguished. The histochemical properties of the E600-resistant esterase investigated are characteristic of cathepsin C. This assumption is strongly supported by the ability of chymotrypsin to hydrolyze *o*-acetylindoxyl. Concerning this substrate, the properties of purified beef spleen cathepsin C are of special interest. This enzyme requires in its substrate a free terminal α-amino or α-imino group. The interaction of cathepsin C with a substrate involves such a group as well as the carbonyl group participating in the sensitive linkage. A primary α-amino group is not essential. Near pH 5 this enzyme shows predominantly hydrolysis, near pH 7·5 predominantly transamidation (Izumia and Fruton, 1956).

With the exception of leucine aminopeptidase, the action of other cathepsins on *o*-acetylindoxyl cannot be wholly excluded at present. Cathepsin B (Greenbaum and Fruton, 1957) must be considered on account of the weak hydrolytic activity of trypsin on *o*-acetylindoxyl and of the activating effect shown by reducing agents. A carboxypeptidase-like action may be deduced from the strong activation by detergents. Organophosphorus compounds do not inhibit purified intracellular proteolytic enzymes. They also fail to influence the precursors of pancreatic proteases in contrast to their strong inhibition of the active enzymes (Jansen *et al.*, 1949). The trypsin-effect on E600-resistant indoxyl esterase suggests that part of this enzyme is present in the cell as a precursor.

A dialkylfluorophosphatase (DFP-ase) (Mounter, Alexander, Tuck and Dien, 1957) seems not to be involved in the enzyme activity described. Hog kidney

DFP-ase acts optimally at pH 8 and is inhibited by dinitrofluorobenzene. On the other hand, the participation of a " C-esterase ", described in hog kidney extract by Bergmann, Segal and Rimon (1957), is most likely. This enzyme differs from the A-esterase of Aldridge and from the DFP-ase of Mounter. It acts optimally at pH 7·4 and is not inhibited by organophosphorus compounds. It is activated by monovalent organic mercurials, an effect which is easily produced with E600-resistant indoxyl esterase. The histochemical evidence, therefore, would suggest a close relationship of C-esterase and a cathepsin, considering the wide pH-range of the latter.

Erythrocytes, plasma and liver esterase hydrolyze *o*-acetylindoxyl at a much higher rate than crystalline chymotrypsin (*cf.* manometric determinations by Underhay, 1957 and Hobbiger, 1957). Quantitative results obtained by purified enzymes or tissue homogenates cannot be related directly to histochemical reactions, since it is not possible to demonstrate a marked difference in enzyme content between various cell types by ordinary biochemical methods.

SUMMARY

The histochemical properties of an organophosphorus-resistant esterase of rat kidney have been studied with *o*-acetyl-5-bromoindoxyl as substrate. The effects of inhibitors and activators, and the determination of the optimal pH, suggest that at least part of this enzymatic action may be due to a cathepsin.

In vitro hydrolysis of *o*-acetylindoxyl by crystalline pancreatic chymotrypsin further suggests that the intracellular proteolytic enzyme involved is probably cathepsin C.

Apart from the kidney, this cathepsin-like activity has been shown in macrophages, Kupffer cells, in various epithelial cells and in the pancreatic islets.

The biochemistry of kidney esterases is discussed in relation to the indoxyl esterase investigated.

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RADIATION LETHALITY IN HISTAMINE DEPLETED RATS

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THE idea that certain reactions caused in tissues by irradiation are due to the release and pharmacological activity of histamine is widely accepted. Ellinger (1951) has marshalled much indirect evidence to support this theory, and drawn attention to certain similarities between the general symptom complex resulting from the systemic effects of irradiation of tissues, the so-called " acute radiation syndrome ", and that seen after the administration of histamine to the intact organism, in anaphylaxis and certain other types of specific and non-specific shock.

Measurements of the plasma histamine and tissue histamine content, after local and total body irradiation have given conflicting results. The plasma histamine has been reported to show some elevation after total body irradiation (Painter, Prosser and Moore, 1947 ; Weber and Steggerda, 1949), whilst tissue histamine has been recorded as unaltered (Ungar and Damgaard, 1954), or lowered (Eisen, Ellis and Wilson, 1956). The generally accepted contention that the tissue mast cell granules are rich in histamine (Riley and West, 1953), and that histamine release agents deplete the tissue histamine largely by their mobilising action on mast cells is supported by both *in vivo* and *in vitro* experimentation and has been reviewed in a recent symposium (Ciba Foundation Symposium on Histamine, 1956).

The present paper primarily deals with the effects of a large dose of total body irradiation given to animals depleted of their skin histamine by 48/80 (MacIntosh and Paton, 1949), over a period, arbitrarily defined as twenty days, and selected to determine the acute lethal effect.

MATERIALS AND METHODS

Adult Canberra black rats were divided into groups of 20 animals, and treated as follows :

Group I received daily intraperitoneal injections of 0·5 ml. saline.

Group II received intraperitoneal injections of Compound 48/80 in saline (100 μg. in 0·2 ml.) in accordance with a chronic dosage schedule used by Feldberg and Talesnik (1953) commencing with 100 μg. twice daily, and increasing each dose by 50 μg. daily till a dose of 450 μg. (twice daily) was reached on the eighth day, and thereafter a single dose of 500 μg. was given daily.

Group III received a daily intraperitoneal injection of 0·3 ml. of promethazine hydrochloride (0·5 mg. per 1·0 ml. of saline).

On the third day of the injections, all animals were subjected to total body irradiation 90 min. after the second daily injection. The animals were irradiated in perspex boxes (3 animals per box) using 250 kV X-rays, and a quality of beam (HVL 1 mm. Cu, 15 MA, FSD 30 cm., dosage rate in air 274 r per min.), and given a dose of 1000 r, calculated with back scatter, at a depth of 2 cm. The irradiation conditions were uniform for all groups.

As animals died in each of the 3 groups, the times of death were recorded, and radiation lethality curves constructed. Previous experiments performed using the chronic dosage

schedule with Compound 48/80, in unirradiated Canberra black rats of comparable weight have shown that the doses employed do not cause death of the animals, although prostration, weakness and other signs of shock usually follow the injections, given over the first 5 days of the course.

In order to determine whether the effect of Compound 48/80 on radiation lethality was due to a radiation protective effect of endogenous histamine in undepleted animals, further groups of 19 or 20 rats were treated as follows :

Group IV received daily intraperitoneal injections of 0·5 ml. saline.

Group V received the same chronic dosage of Compound 48/80 as Group II above, but the injections were commenced 24 hr. after irradiation.

Group VI received two single doses of Compound 48/80, each of 200 μg., intraperitoneally on the day of irradiation, the second injection being given 90 min. before irradiation.

Each group received 1000 r total body irradiation, using identical factors and technique as for Groups I–III. Lethality amongst the animals was charted daily.

Since cortisone and substances with similar action are known to affect shock-like conditions the effect of cortisone acetate and of piromen on acute radiation lethality were examined in another series of experiments, again using 3 groups of 20 rats. Piromen, a polysaccharide prepared from a *Pseudomonas* species, is pyrogenic and has ACTH-like properties (Soylemezoglu and Wells, 1951). It reduces acute lethality from traumatic shock and burns in rats (Greene, Stuart and Joralemon, 1953), causes degranulation of mast cells (Stuart, 1950) and presumably depletion of tissue histamine. The groups were treated as follows :

Group VII received daily intraperitoneal injections of 1·0 ml. buffered saline (pH 6·8).

Group VIII received a daily intraperitoneal injection of 2 μg. piromen in 1·0 ml. buffered saline (pH 6·8).

Group IX received daily 3 mg. cortisone acetate diluted in 0·3 ml. normal saline intramuscularly.

Ninety min. after the second injection, all animals received 1000 r total body radiation. Lethality, incidence of the onset of diarrhoea and loss of body weight were recorded.

RESULTS

Effect of histamine depletion on radiation lethality

The results are recorded in Fig. 1 and 2 and the details of the various experiments using Compound 48/80, promethazine hydrochloride, piromen and cortisone acetate, are set out in Table I together with the statistical evaluations.

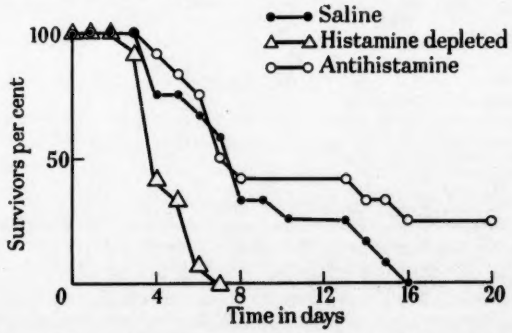

FIG. 1.—Survival curves for groups of rats, all subjected to 1000 r total body irradiation (250 kV X-rays, HVL 1 mm. Cu, 15 MA 30 cm. FSD and dosage rate 274 r/min. in air). Individual groups treated with saline, Compound 48/80 and promethazine hydrochloride respectively.

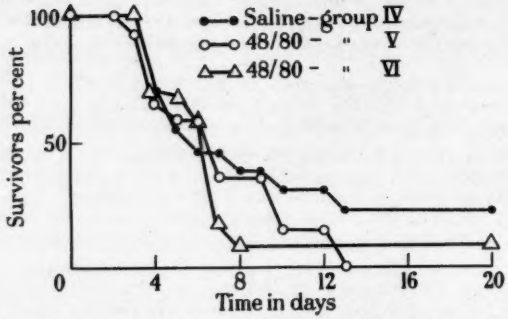

FIG. 2.—Survival curves for groups of rats subjected to 1000 r total body irradiation, and treated respectively with saline, chronic depletion with 48/80 commenced 24 hr. after irradiation and acute depletion with 48/80 preceding irradiation.

TABLE I

Experiment	Group	Number of animals*	Mean weight (±S.D.)†	Mean survival over 20 days (±S.D.)	Significance‡
1	I—Control (saline)	20	117 (±9)	8·67 (±4·10)	—
	II—Chronic depletion with 48/80 commenced before irradiation	20	118 (±15)	4·91 (±1·04)	$t=3·8$ $p=0·001$
	III—Antihistamine (promethazine HCl)	20	114 (±10)	11·17 (±6·09)	$t=1·5$ $0·2>p>0·1$
2	IV—Controls (saline)	19	130 (±14)	9·31 (±5·92)	—
	V—Chronic depletion with 48/80 commenced after irradiation	20	139 (±20)	7·21 (±3·45)	$t=1·3$ $p=0·2$
	VI—Acute depletion with 48/80 immediately preceding irradiation	19	141 (±17)	7·08 (±4·25)	$t=1·3$ $p=0·2$
3	VII—Controls (saline)	20	195 (±30)	12·92 (±4·79)	—
	VIII—Piromen (daily injections before and after irradiation)	20	198 (±23)	12·08 (±6·61)	—
	IX—Cortisone acetate (daily injections before and after irradiation)	20	191 (±34)	11·58 (±5·66)	—

* Litter mates were equally apportioned to the groups in each experiment.

† The variation in survival rates for control groups is due to differences in body weight in the groups. For the irradiation factors used throughout in these experiments, the mean survival in days (y) determined for Canberra black rats of body weight, (x) g. using the above data, together with other data for similar laboratory stock rats, is essentially a linear relationship over a range in body weight from 100–250 g. This relationship has been determined by the least squares method and is given by a regression line, $y = 0·054x + 2·292$, from a mean dose of 1000 r in tissue.

‡ The distribution (t) has been calculated using Bessel's correction $\left(\frac{n}{n-1}\right)$ in determining the standard error of the difference of the means, $\left(\hat{\sigma}_{\omega=}\hat{\sigma}\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}\right)$

It will be seen that the mean survival of the saline injected control animals, after a lethal dose of total body irradiation, exceeded that of the 48/80 treated animals. The increased survival of the animals receiving the antihistamine, promethazine hydrochloride, is not statistically significant ($p > 0{\cdot}1$).

Chronic histamine depletion with Compound 48/80 did not increase radiation survival in the rats, in fact the mean survival was decreased. The effect was more pronounced when histamine depletion was commenced prior to irradiation (Group II) than after irradiation (Group V). In Group II there was a highly significant increase in the lethality ($p < 0{\cdot}001$), the average survival of animals over the arbitrary twenty-day period being halved.

Acute histamine depletion with Compound 48/80 before irradiation, so that irradiation was administered to an animal whose tissues were greatly deficient in histamine (Group VI), caused an increase in lethality, although strict statistical comparisons of the distribution of survival times fails to establish a satisfactory significance.

Post mortem studies of depleted animals and undepleted animals which died after irradiation failed to establish significant differences in post mortem appearances, except that oedema of subcutaneous tissues and peritoneal exudation were much more prominent in the animals treated with Compound 48/80. The incidence of diarrhoea before death was equal in both depleted and undepleted animals, and generalised bacteraemia—a constant post mortem finding in rats dying from irradiation shock—was evident in both groups.

Treatment of animals with cortisone and piromen (Groups IX and VIII respectively) failed to influence acute radiation lethality (Table I) and loss of weight after irradiation (Table II). However, cortisone treatment delayed the appearance of diarrhoea in irradiated animals (Table II).

TABLE II

Group	Mean weight (g.) of animals before irradiation	Mean weight (g.) of surviving animals six days after irradiation	Incidence of diarrhoea (per cent animals in group) in third day after irradiation
VII—Controls (saline treated)	195	144	42
VIII—Piromen treated	198	155	25
IX—Cortisone treated	191	140	0

DISCUSSION

The relative increases in lethality in histamine depleted groups suggests that the significant factor which governs this increase seems to reside in the completeness of histamine depletion rather than in the levels of tissue histamine which prevailed at the time of irradiation. In animals of Group VI, the largest tolerable doses of Compound 48/80 were given to cause a profound lowering of tissue histamine levels (Riley and West, 1955), the animals being allowed to recover from shock prior to irradiation to ensure that the histamine which was released had largely disappeared or been inactivated. The latter claim is supported by pharmacological studies; in rats, measurements of the rise in plasma histamine, fall in tissue histamine and the urinary excretion of histamine following a single injection of Compound 48/80 all point to the conclusion that within an hour of an intraperitoneal injection an equilibrium state is reached, and the concentration

of histamine in the tissues is lowered (see Wilson, 1954; Mongar, 1956; Paton, 1951). However, to be taken into consideration is the fact that chronic depletion of an animal causes bursts of histamine release at decreasing levels, which markedly affect the physiological equilibrium and cause considerable stress in the treated animals for a short period after injection. In the heavily irradiated animals, in these experiments, early injections of 48/80 still elicited manifestations of shock, although less severe than in unirradiated animals. This added insult to physiological mechanisms, already severely compromised by heavy total body irradiation, is considered to play a significant role in the increase in lethality observed in such animals. However, the decrease in survival time of acutely depleted Group VI animals in which periodic bursts of histamine release did not take place after irradiation, but in which tissues still remained depleted of histamine for a considerable time (6 weeks) after the injection (Feldberg and Talesnik, 1953), suggests lack of readily available tissue histamine jeopardises recovery after total body irradiation. Whilst the function of histamine in the intact organism is still something of an enigma, it would be surprising if this prominent component of tissues did not serve a protective function in injury, rather than behave as an endogenous poison responsible for vascular damage and cell death.

The actual increase in lethality which followed irradiation of rats, acutely depleted of histamine and irradiated whilst tissue histamine was greatly decreased, seems to support the claim that histamine is a true radiation protective substance *in vivo* (Bacq and Herve, 1955), similar to many other amines. These authors record a 23 per cent protection against radiation lethality for histamine, although results were stated to be very variable. Antihistamines have been reported to act in an opposite manner, and increase radiation lethality (Halpern, Cuendet and May, 1942; Hervé and Lecomte, 1955). These findings are difficult to accept on certain grounds. Firstly, the thoroughness of histamine depletion, rather than the tissue histamine content at the time of irradiation, seems the crucial factor in animal survival in the experiments which have been recorded in this paper. Secondly, administration of promezathine hydrochloride, a potent antihistaminic, did not increase radiation lethality, but if anything improved animal survival. Finally, the beneficial effect of antihistamines in the symptomatic treatment of radiation sickness is well attested. Whilst an antihistamine has several side effects quite apart from a specific antihistamine action, e.g., atropine-like effects, there is no evidence that its administration substantially reduces the tissue histamine content, although certain antihistamines have been shown to exhibit histamine releasing properties (Schild, 1956). However, it is very unlikely that an antihistamine would act indirectly as a radiation protective agent by causing a substantial release and depletion of the protective amine during irradiation.

The finding that continued cortisone administration has an anti-inflammatory action and decreases tissue damage after local irradiation (Houghton, Walter and Jones, 1954) warrants consideration from the aspect of histamine activity and release.

The effect of cortisone on histamine release and availability is still largely undecided (see discussion on Schayer, 1956). The concensus of opinion seems to be that cortisone does not prevent release of histamine but prevents the action of histamine released by injury or a depleting agent. Furthermore, it prevents the replacement of discharged tissue histamine and rebinding of such histamine

to cell depots (mast cells). There is apparently little information available as to whether cortisone treatment *per se* alters tissue histamine levels. Indirect evidence available relates to the effect of systemic and topical cortisone therapy on the mast cells in the rat. Zachariae and Moltke (1954) reported no quantitative changes, but some alteration in mast cell morphology, a finding duplicated by Smith and Lewis (1954), and supported by results obtained by the writer.

If cortisone prevents the action of histamine released by irradiation, and if acute radiation lethality is governed by the histamine released, it would be reasonable to expect a substantial alteration in survival amongst cortisonised animals. The experiments performed fail to realise this expectation, either using cortisone or piromen, which has certain cortisone- and ACTH-like properties, besides inducing mast cell changes. The fact that cortisone reduced gastro-intestinal manifestations of radiation damage whilst 48/80 did not, is interesting in view of the finding that gastro-intestinal mucosa is poor in mast cells, and in that its histamine content is not reduced by 48/80. In so far as gastro-intestinal damage and endogenous spread of infection play such important roles in the acute radiation syndrome and radiation lethality, a failure to release mucosal histamine with depleting agents must be taken into account in interpreting experimental results. The effect of cortisone on the concentration and release of histamine from gastro-intestinal mucosa similarly warrants consideration.

SUMMARY

Acute radiation lethality in rats was found to be increased if the animals were treated with Compound 48/80, to reduce the tissue histamine. This increase in lethality was greatest with chronic depletion of tissue histamine, whilst acute depletion, prior to and during irradiation, had less effect on radiation lethality.

Promethazine hydrochloride, piromen and cortisone acetate individually failed to influence significantly acute radiation lethality in rats.

The increase in radiation lethality observed in 48/80 treated animals is considered due to lack of available histamine, in the post irradiation period, as an endogenous metabolite essential in tissue recovery. The view that histamine and related amines have a radiation protective effect which operates at the time of radiation exposure, is not supported by the difference in results obtained following acute and chronic histamine depletion with 48/80.

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THE ABSORPTION OF *CLOSTRIDIUM BOTULINUM* TYPE A TOXIN FROM THE ALIMENTARY CANAL

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THE orally toxic nature of *Clostridium botulinum* type A toxin towards many species, including man, has been known since the work of Van Ermengem (1896). Its peculiar interest lies in the fact that the toxin appears to be a protein and would be expected to undergo proteolytic digestion in the alimentary canal as happens with tetanus and diphtheria toxins. This does not occur and a number of experiments to determine the site of absorption have been described. Dack (1926) and Dack and Gibbard (1926*a*, 1926*b*, 1925–26) showed that toxin was absorbed from the perfused small intestine of the hog and rabbit as demonstrated by the toxicity of the perfusate to mice. Dack and Hoskins (1942) demonstrated some absorption from colonic pouches in Macaca mulatta but much larger quantities of toxin had to be used than were required to cause death following oral administration. Coleman (1954), using dogs, was able to demonstrate only very small amounts of toxin in the blood stream after putting crystalline toxin into the ileum. These experiments suggested the upper small intestine, and possibly the stomach, as the most important regions of the gut for toxin absorption.

In the present experiments absorption from the stomach and small intestine has been tested, mainly using rabbits. With rats, evidence has been obtained that absorbed toxin appears first in lymph and only secondarily in the blood. Alterations in the feeding habits of mice have been made in an effort to change the oral toxicity as compared with normal animals.

A preliminary account of some of this work has been given (May and Whaler, 1956).

METHODS

Toxin production.—Cl. botulinum type A, strain 4587, was grown at 34° for 3 days in a modified CCY medium (25 per cent tryptic digest casein, 10 per cent yeast extract in water : 1 per cent glucose added after autoclaving). At the end of incubation a further 1 per cent of glucose was added and the culture allowed to stand at room temperature for 10 days. In this time autolysis took place and a high proportion of the toxin present in solution was adsorbed on the bacterial debris. From a litre bottle of this culture 100 ml. lots were dispensed into small containers and subsequently stored at 4° until required. Potency was retained for at least 18 months. This crude whole culture was used in most experiments and contained $0{\cdot}8–1{\cdot}2 \times 10^6$ mouse units/ml. (defined below). Where necessary, a high degree of purification was obtained by extracting the bacterial debris with citrate buffer pH 3·8–4·0, precipitation with ammonium sulphate (30–40 per cent saturation) followed by solution in acetate or phosphate buffers and further ammonium sulphate fractionation. This method, based on a procedure of Bottomley and Creeth (unpublished) and briefly described by Halliwell (1954) gave toxin of high specific activity (1 mg. N = $1{\cdot}7 - 2{\cdot}2 \times 10^8$ mouse units) although some batches proved difficult to purify to this state.

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Assay of toxin.—Serial dilutions (× 1·3) of toxin in 0·2 per cent gelatin buffered with 1 per cent $Na_2HPO_4.12H_2O$ and concentrated HCl to pH 6·6 (after Lamanna and Glassman, 1947) were made and 0·5 ml. of each dilution given intraperitoneally to a group of ten or twenty 18–22 g. male mice (Porton strain). Mortality over 7 days was determined and the LD_{50} dilution with 95 per cent fiducial limits calculated using probit analysis (Finney, 1952). Except where stated otherwise, amounts of toxin are referred to in mouse units/ml., one mouse unit being equivalent to 1 LD_{50} determined as above. In some assays groups of 5 mice only and a × 2 dilution factor were unavoidable, when the results obtained gave only approximate values of toxicity. The toxicity to rabbits was determined in a similar way but using 1·0 ml. as the test volume of diluted toxin.

The above method was used to determine the titre of whole culture or partially purified toxin, and also samples of blood etc. taken from experimental animals. That lethality of the samples was due to the toxin and not to other causes, *e.g.*, bacterial contamination, was checked occasionally by injecting the diluted samples into 2 groups of mice, one group having had a previous (2 hr.) injection of horse antitoxin. Protection was invariably obtained.

Feeding experiments.—Rabbits and mice were used to determine the oral toxicity and for a comparison with the i.p. titre. Rabbits were given intravenous thiopentone sodium (Abbott Laboratories Ltd., 50 mg./ml.) until the animal was completely relaxed. A polyethylene tube was passed into the stomach and 10 ml. toxin given, followed by 2–3 ml. of air to empty the tube of toxin before withdrawal. The animals recovered from the anaesthetic in 5–20 min. Unanaesthetized mice received (usually) 0·25 ml. toxin through a No. 22 gauge ball-tipped needle passed down the oesophagus into the stomach. In both cases × 2 serial dilutions of toxin were given and the oral LD_{50}, with limits, determined using 4–7 groups of 8–20 animals each. At the same time the i.p. titre was determined. Deaths from the *per os* administration of saline or dilute toxin (*ca.* 100 mouse units/ml.) were less than 1 per cent and no correction was made for this in the results. The efficacy of oral toxin was assessed as the ratio of the number of mouse units in one oral LD_{50} to the number of mouse units for one i.p. LD_{50}.

In the rat experiments the animals were given 5–20 per cent whole culture toxin, diluted with 0·2–0·9 per cent saline, as drinking fluid. The ingested dose was calculated from the toxin titre and the volume consumed.

Animal preparations.—Rabbits were anaesthetized with pentobarbitone sodium (32–40 mg./Kg., Abbott Laboratories Ltd.), the abdomen opened along the mid-line and the desired section of the gut isolated. In initial experiments the stomach, upper 30–40 cm. of small intestine or the lower ileum was isolated by ligature. Toxin was then injected through the gut wall into the lumen using a syringe and fine needle. In later experiments, to prevent the development of excess pressure within the lumen and to ensure complete absence of contamination of the peritoneal and serosal surfaces by toxin, glass cannulae were tied into the proximal and distal ends of the region selected. When the precise site of absorption was not our main interest a small cannula was inserted through the intestinal wall and securely tied without occluding the lumen of the intestine. In all cases care was taken to avoid haemorrhage, to retain a good blood supply to the region being studied, to prevent pressure within the segment and prevent serosal or peritoneal contamination with toxin. Experiments where any doubt existed on the above points were discarded.

After such operations, which were made under deep anaesthesia, the abdomen was sewn up and light anaesthesia maintained throughout the experiment.

When required, the thoracic lymph duct was cannulated close to its entrance into the left caval vein using a small stainless steel or polyethylene cannula. In some cases cannulation of the duct was not possible and lymph was allowed to drain from the cut end of the duct into a small depression in the neck and collected periodically with a dropping pipette. Clotting was prevented by the occasional application of solid heparin. Lymph flows varied from 0·5–8·0 ml./hr. and were maintained from 3–10 hr.

Rats were anaesthetized with ether and the main lymphatic vessel draining the intestine cannulated. Any other lymph vessels (usually only one was seen) were tied off. Polythene tubing size 46 or 47 (Portland Plastics Ltd.) was used and the method described by Bollman, Cain and Grindlay (1948) was followed. The operated animals were allowed to recover from the anaesthetic and kept in restraining cages for 2–6 days. Food was allowed at all times and the animals were given 0·2–0·9 per cent saline or saline-diluted toxin to drink. Lymph collection was continuous, and samples were assayed for toxicity on rats and mice.

RESULTS

Site of toxin absorption

Table I summarises the results of 32 experiments on rabbits of which 12 are from animals where intestinal cannulae were not used. The results of these

TABLE I.—*Absorption of Toxin from the Gastro-intestinal Tract of Rabbits*

Region	Terminal plasma: Number of animals	Terminal plasma: Number giving toxic plasma	Terminal plasma: Highest plasma titre (mouse units/ml.)	Thoracic duct lymph: Number of animals	Thoracic duct lymph: Number giving toxic lymph	Thoracic duct lymph: Highest lymph titre (mouse units/ml.)
Stomach	11	2	*ca.* 1	4	0	—
Upper small intestine	13	5	*ca.* 8	13	10	*ca.* 120
Lower small intestine	8	4	*ca.* 2	3	0	—

In each experiment 2×10^6–4×10^7 mouse units in crude culture were given in small lots over a period of 1–6 hr. Duration of experiments 3–10 hr.

twelve were similar to the remaining 20 experiments using cannulae and have therefore been combined. As demonstrated by the presence of small amounts of toxin in terminal plasma absorption from the lower half of the small intestine occurred in 50 per cent of the tests and from the stomach in 2 of 11 experiments. In 7 of the experiments in these 2 groups lymph was collected but no toxin could be demonstrated in it. The upper half of the small intestine was much more permeable and toxic lymph was obtained from nearly all of the animals tested and in some cases, terminal plasma was also toxic though to a level below that of the lymph.

TABLE II.—*Toxicity of Lymph During Absorption from the Upper Small Intestine*

Expt.	Lymph sample No.	Vol. of sample (ml.)	Collection time (min.)	Approx. titre (mouse units/ml.)	Total toxicity (mouse units)	Total toxin given during the collecting period (mouse units)
I	1	2·6	55	0	0	0
	2	5·7	120	3	17	8×10^6
	3	5·4	60	6	33	2×10^6
	4	8·5	100	16	135	$2{\cdot}5 \times 10^6$
	5	4·3	60	12	50	0
	6	5·6	120	16	90	4×10^6
	7	4·8	60	8	38	0
					Total = 360	
	Terminal plasma contained 2 units/ml.			Approx. plasma vol. = 95 ml.		
II	1	4·0	35	0	0	0
	2	10·2	150	4	40	5×10^6
	3	17·5	225	120	2100	18×10^6
					Total = 2140	
	Terminal plasma contained 8 units/ml.			Approx. plasma vol. = 120 ml.		

Detailed results from two experiments are given in Table II. In each one lymph was collected continuously and toxin given in small lots ($1–6 \times 10^6$ mouse units) at approximately hourly intervals. Total toxin given in any one collection period is shown along with the volume and titre of the collected lymph.

In experiment 1, Table II, the total circulating toxin at the end of the experiment is approximately 190 mouse units and in experiment 2, Table II, about 960 mouse units, in both cases about 50 per cent of that recovered in the lymph. Results presented below suggest that it is unlikely that more than 190 or 960 mouse units respectively were lost from the circulation during the experimental period, therefore in these two experiments something like 50 per cent of the absorbed toxin was collected from the thoracic lymph duct.

At the end of these experiments the experimental section of the gut was washed thoroughly with buffer and the washings collected. Large particles were removed by filtration through gauze and the filtrate assayed for toxicity. Two to 30 per cent of the toxin given could be recovered : some may have been adsorbed on to food particles and it is assumed that the remainder had been absorbed or destroyed.

A few experiments were carried out on anaesthetized rats, terminal plasma being tested for toxin after 2–5 hr. by injecting 1·0 ml. i.p. into each of 5 mice. No absorption from the stomach or caecum was detected (3 rats in each group) but terminal plasma was toxic in all 3 test animals after putting toxin into the upper small intestine and in 1 of 3 animals when toxin was in the lower half of the small intestine.

Diffusibility of toxin in lymph

Toxic rabbit lymph (*ca.* 120 mouse units/ml.) was dialysed at 4° against 2 vol. saline for 96 hr. and for some months. No toxicity appeared in the diffusate and the indiffusable material retained at least 20 per cent of its toxicity. In this experiment we could have detected toxin in the diffusate if only 2–4 per cent of that present in the dialysing sac had diffused through.

Route of toxin absorption

The rabbit experiments showed that a considerable percentage of toxin which penetrated the intestinal mucosa was transported in the lymph stream. Experiments with unanaesthetized rats were used to determine whether the toxin was primarily absorbed into the lymphatic vessels. It seemed reasonable to assume that if rats were completely protected from toxaemia by the removal of intestinal lymph to outside the body then the lymphatic route could be regarded as primary in the absorption of toxin.

Rats were used in which the main intestinal or thoracic lymph duct was cannulated. They consumed toxin in their drinking fluid, the intake of which varied in volume from 20–120 ml./24 hr. Lymph flows of similar rate were obtained in different experiments. In those animals (see below) where the cannula was blocked fluid consumption was reduced. The results fell into 3 groups.

Group I.—Eight rats, which over a period of 12–48 hr. consumed 0·7–24·0 $\times 10^6$ mouse units of crude culture toxin (*ca.* 3–110 *oral* rat LD_{50}) gave good flows of toxic lymph and did not die. Most of the toxic lymph was collected during and in the 24 hr. following toxin ingestion and in total, 12–234 mouse units were collected from individual animals, more than sufficient to kill a rat by i.p. injection, the LD_{50} dose for which is 10–25 mouse units. Some of the rats showed evidence of botulism but all recovered in 3–10 days.

Group II.—A further 11 animals were similarly prepared, but after a few hours the cannulae became blocked due to clotting of the lymph or were deliber-

ately closed off with forceps as soon as the rat had consumed a sufficient quantity of toxin. These animals consumed $0{\cdot}7–13 \times 10^6$ mouse units of toxin and all died within 48 hr. (range 18–48 hr., mean 29 hr.).

Group III.—Six rats, in spite of a good lymph flow, died after ingesting $1{\cdot}7–14 \times 10^6$ mouse units. Death occurred later than in those animals of Group II having a range of 48–60 hr. and a mean value of 53 hr., significantly longer ($P = 0{\cdot}001$) than for Group II. In these 6 animals and in a number of others which gave good lymph flows and survived for 3–7 days (these have not been included in the group average) lymph titres were high and in one case exceeded 24 mouse units/ml. 1·0–2·5 ml. of toxic lymph from the 6 animals was injected i.p. into normal rats and in 5 cases the injected animals died before the donor animal, *i.e.*, in 18–48 hr.

Removal of circulating toxin from the blood stream

In all of the experiments where terminal plasma was toxic the titre never exceeded 8 mouse units/ml., and was usually lower, although lymph titres up to 120 mouse units/ml. were obtained. This difference could be due to the slow rate of entry of toxin into the blood or by its very rapid removal due to destruction, excretion, or absorption by tissues. We have followed the rate of removal of toxin from the blood following i.v. injection of whole culture toxin diluted 1000 × with saline prior to injection. Ideally toxin which had been absorbed from the gut should have been used but insufficient was was available.

Rabbits were given i.v. approximately 2000 mouse units of toxin. In some experiments blood samples were taken at 3–10 min. and again after 4–8 hr.: in others half of the injected rabbits were killed after 3–10 min. and the remainder after some hours. Always the initial blood sample was approximately as calculated from the assumed blood volume and the dose of toxin given. The results given in Table III show that the rate of removal from the blood stream was slow, but measurable.

TABLE III.—*Removal of Circulating Toxin from the Blood Stream*

Expt.	Initial blood titre (mouse units/ml.), with 95 per cent limits	Final blood titre (mouse units/ml.), with 95 per cent limits	Duration (hr.)	Relative potency of terminal* blood, with 95 per cent limits
1	13·6–12·6–11·5	11·7–10·9–10·1	4·0	0·77–0·87–0·97
2	17·4–15·8–14·7	12·9–11·9–11·1	6·5	0·67–0·75–0·81
3	19·0–17·3–15·7	9·4– 8·7– 8·0	8·0	0·44–0·50–0·57

* Initial blood level = 1·0.

Urine has been assayed for toxicity on a number of occasions with negative results. A single experiment to determine the rate of inactivation of toxin by rabbit urine *in vitro* showed that less than 50 per cent of the toxicity disappeared in 4 hr. after incubation at 37° and urine pH = 7·5. It would appear that at most only very minute amounts of toxin can be excreted by the kidney.

Oral toxicity to mice, rats and rabbits

The oral/i.p. ratio for rabbits using crude whole culture was determined in 2 experiments. In the first a ratio of *ca.* 1000, and in the second a ratio and limits of 820–1220–1810, were obtained. The LD_{50} (i.p.) for rabbits is 25–40 mouse units. A single experiment using rats gave a ratio and limits of 8200–16,800–34,600.

The oral/i.p. ratio in mice has been determined more freqently and for whole culture is 27,100–31,100–35,800 (mean and limits of 7 experiments). Two samples of relatively pure toxin (specific activities 1·9 and 2·0 $\times$ 10^8 mouse units/mg. N) gave ratios and limits of 21,760–33,620–51,950 and 53,950–73,030–98,860 respectively. The latter figure suggests that some small decrease in the oral effectiveness of the toxin has occurred during purification. On a weight basis mice were about 4 times less sensitive to i.p. toxin and 100 times less sensitive to oral toxin than rabbits.

Using whole culture, no significant difference in the response of mice of weights ranging from 15–35 g. was demonstrated. Similarly using a standard dose of toxin contained in 0·05–0·4 ml., the volume of toxin given by stomach tube did not affect the result.

The effects of short fasts were studied using mice. After being deprived of food for 20–22 hr. mice were given toxin by stomach tube, and allowed to feed immediately or some time afterwards. The results are given in Table IV. The control oral LD_{50} has been given a relative potency of 1·0 and the other results are expressed in terms of this unit. A relative potency > 1 shows that the given dose of toxin was more effective than the same dose to a normal mouse. Fasted animals given access to food at the time of toxin administration were more susceptible than controls or fasted mice where food was withheld for a further 3–4 hr. In all groups the i.p. response was not affected.

TABLE IV.—*Effect of Fasting upon Oral Toxicity in Mice*

Expt.	Mouse group	Relative potency of toxin with 95 per cent limits	Remarks
1*	1	1·0	Normal controls.
	2	1·33–2·32–4·07	Food withheld 20 hr., allowed to feed 1 hr., given toxin and food after a further 1 hr.
	3	1·39–2·46–4·32	Food withheld 22 hr., then given toxin and allowed to feed.
	4	0·57–0·97–1·63	Food withheld 22 hr., then given toxin. Food allowed after a further 3 hr.
2	1	1·0	Controls: food withheld 75 min., then given a small cube of toxin-contaminated food.
	2	1·6–2·6–4·8	Food withheld 7 hr. then as group 1.

* Two other experiments gave similar results.

In experiment 2, Table IV, the standard doses of toxin were put on to small cubes of food. There was a significant difference between fed and fasted mice. The result is in line with that of experiment 1, Table IV, but since we could not be absolutely certain that the control group of mice (in which the desire to feed would presumably be less than in the experimental group) ate the whole of the prepared cube, too much emphasis cannot be given to this result.

DISCUSSION

The site of toxin absorption from the alimentary canal has been studied previously. From a number of experiments Dack and Gibbard (1925–26, 1926*a*) concluded that in rabbits, guinea-pigs and perhaps hogs absorption was mainly

from the caecum and small intestine. Some absorption from the colon, but a much greater permeability of the stomach, small intestine or caecum, was suggested from experiments using Macaca mulatta (Dack and Hoskins, 1942). Coleman (1954) showed that some toxin was absorbed from the ileum of the dog. Considerable species differences in susceptibility to the oral ingestion of type A, and also types B–E, toxins exist, and a number of tests on the oral/i.p. ratio have been made (Legroux and Levaditi, 1947 ; Moll and Brandley, 1952).

The present experiments show that the upper small intestine was the most important site of toxin absorption in rabbits. In a number of cases toxin was absorbed from the ileum and in 2 of 11 experiments from the stomach. The caecum and colon were not tested in this species but in the rat, absorption from the caecum (and stomach) was not sufficient to give demonstrable toxin in the blood.

During all these experiments we were very conscious of the extreme sensitivity of our test for the detection of toxin. The blood and lymph titres which we obtained were very small in comparison with the doses used, and as little as 1 μl. of our toxin (equivalent to 1×10^3 mouse units) would, if it entered the circulation, give an average plasma titre of 3·8 mouse units/ml., *i.e.*, as large as those we obtained in our experiments. For this reason extreme care was taken in all our animal preparations and experiments were discarded where any chance contamination of peritoneal or serosal surfaces was suspected. Because of these precautions we feel that the results of toxin absorption presented here, although small in amount as expressed in terms of N absorption or as a fraction of the experimental dose, represent real findings. In rabbits, although the experimental preparation and technique for each of the regions of the gut studied were similar, the results had a definite grouping. In addition the results of rat experiments, where toxin was put into a specific region of the alimentary canal, are also grouped and of these in two regions (stomach and caecum) no demonstrable absorption occurred.

In many of the rabbit experiments thoracic duct lymph gave a higher toxin titre than did terminal blood. It is unlikely that these high titres derived from low-titre blood (see Yoffey and Courtice, 1956) and more probable that much, and possibly all, of the absorbed toxin entered the blood stream from the lymphatics and not directly from the intestine. Although lymph titres were sometimes lower than those found in the blood, and by calculation it is clear that the total circulating toxin at the end of some of our experiments exceeded the total collected from the thoracic duct, these facts do not offer insuperable difficulties to the hypothesis, for a number of reasons: (*a*) veno-lymphatic anastomoses are occasionally demonstrated in animals and may be very frequent, (*b*) the thoracic duct frequently enters the superior caval vein by multiple openings, one or more of which may remain open during an experiment and (*c*) intestinal lymph can be carried in the right lymph duct. In any of these states, toxic lymph would enter the bloodstream.

Objection might also be made that absorbed toxin was very rapidly removed from the blood, and that our terminal blood toxin represented only a small fraction of that absorbed. We have no information on the rate of removal of absorbed toxin from the blood but crude culture toxin given i.v. was only slowly removed (Table III, see also Dack and Gibbard, 1925–26 ; Coleman, 1954). If we accept this removal rate as representative of the situation in our experiments we would expect, in a 6–8 hr. period, that the final circulating toxin would represent about 50 per cent of the maximum which could have been present. In experiment 1,

Table II, lymph-borne toxin was > 360 mouse units and final total blood toxin was 190 mouse units. If this latter represented 50 per cent of that ever present, the remaining 50 per cent having been removed from the blood as Table III would suggest, then the maximum circulating toxin was 380 mouse units. Therefore in this experiment (and a similar result was obtained from experiment 2, Table II) at least 50 per cent of the total absorbed toxin had entered directly into the lymphatic vessels.

The rat experiments provided further evidence that absorbed toxin entered primarily into the lymphatics. By cannulation of the main intestinal lymphatic or the abdominal thoracic duct it was possible completely to prevent death in some animals ingesting toxin or, in other cases, to prolong considerably their life as compared with controls in which the cannula was blocked. High-titre lymph collected in many of these experiments and given i.p. to normal rats caused death from botulism and, in many cases, these rats died *before* the donor animals.

During the last decade botulinum type A toxin has been purified and shown to be a protein of high M. Wt. ($0{\cdot}9$–$1{\cdot}13 \times 10^6$) (high specific toxicity to mice) 1 mg. N = 220–260×10^6 mouse i.p. LD_{50}) and of normal amino-acid composition (Lamanna, McElroy and Eklund, 1946; Abrams, Kegeles and Hottle, 1946; Buehler, Schantz and Lamanna, 1947; Duff, Wright, Klerer, Moore and Bibler, 1957). Attempts to obtain toxic sub-units of low M. Wt. have been made by Wagman and Bateman (1953) and Wagman (1954) under defined conditions of pH and ionic strength. Fractions with a specific toxicity 2–3 times that of their starting material have been prepared but no evidence for a M. Wt. less than 40,000 has been obtained. Bronfenbrenner and Schlesinger (1921–22) and Rodopulo (1941) have claimed that the toxin was dialysable and would therefore be of a lower M. Wt. than others have found. Precise experimental conditions were not given in these reports. Using a variety of solvents, over a pH range of $3{\cdot}8$–$7{\cdot}5$, we have obtained no evidence that our preparations were dialysable and we assume that in our experiments and also in some cases at least of natural botulism a protein molecule is being absorbed. To some extent our dialysis experiments with toxic lymph confirm this, although the possibility of a small toxic unit adsorbed onto plasma protein cannot be discarded. If we accept that botulinum toxin is a protein molecule our results have some relevance to protein absorption in general.

Using guinea-pigs, Hettwer and Kriz-Hettwer (1925, 1926) showed that small amounts of horse serum protein were absorbed from the small intestine, and that an increase in intra-lumen pressure facilitated this absorption. This latter effect may have some importance in naturally occurring botulism as we invariably found distended intestines in rabbits immediately after death from ingested toxin. Harten, Gray, Livingstone and Walzer (1941) demonstrated the absorption of raw cotton-seed protein from the stomach and oesophagus of monkeys by skin sensitization reactions, and certain food allergies may represent similar phenomena. Only very small amounts of protein are needed to produce such reactions. In our experiments the absorption of 5–60 ng. of toxic protein would be sufficient to cause death. With the exceptions noted below, protein absorption probably occurs only to a very slight degree and normally identification would be difficult. The high specific toxicity of botulinum toxin makes detection of small amounts a relatively simple matter.

The absorption by new-born calves of large amounts of immune colostrum globulin by the lymphatic route has been shown by Comline, Roberts and Titchen

(1951) and in many respects these studies provide a parallel with the rat experiments described herein. Further evidence that the lymphatics draining the small intestine carry absorbed protein is given by Alexander, Shirley and Allen (1936) who showed that egg white protein given by stomach tube to dogs rapidly appeared in the thoracic duct lymph although it could not be demonstrated in portal blood. The lymphatic route for the absorption of protein has not been examined, as far as we know, by any other worker.

Most of the ingested toxin in our experiments was not absorbed. In the rabbit only 0·1 per cent of the ingested dose was absorbed and the amount was even less in the mouse. Although early reports (Schubel, 1923; Bronfenbrenner and Schlesinger, 1924; Dack and Gibbard, 1926) suggested that crude toxin was resistant to the gastric and intestinal secretions, some proteolytic digestion of the toxin must occur, as later work (Halliwell, 1954; Coleman, 1954) has shown that some destruction of fairly pure toxin by pepsin and more by chymotrypsin and trypsin occurs *in vitro*.

There is considerable disagreement between the early studies using crude toxin and few animals for bio-assay and the results of Coleman (1954) and especially Halliwell (1954) who used fairly pure toxin preparations and more accurate methods of bio-assay. Coleman attempted to reconcile these differences by postulating that crude toxin contained an oral potentiating factor which was removed during purification. The results of only two experiments using fairly pure toxin do not wholly support this concept, but strain and culture medium differences may give rise to considerable variation in results (*e.g.*, see Duff *et al.*, 1957, on the difficulties of obtaining pure toxin following repeated sub-culture of the stock strain).

The factors responsible for the difference in oral sensitivity between mice and rabbits are unknown but the nature and concentration of proteolytic enzymes, variations in the movement of food or differences in mucosal permeability are all possible. So far attempts to analyse the factors responsible for species differences in oral toxicity have not been reported.

Somewhat surprising is the increased effectiveness of oral toxin if feeding takes place at the time of toxin ingestion. Stimulation of the secretion of proteolytic enzymes during feeding might be expected to reduce oral potency. This does not happen, evidence perhaps that the mucosal membrane becomes more permeable following feeding. It might be noted that the enhanced toxicity accompanies a meal taken after some hours of fasting, the conditions under which humans and many animals would be most likely to ingest toxin.

SUMMARY

The absorption from the alimentary canal of *Cl. botulinum* type A toxin has been studied in rats, rabbits and mice. In rats and rabbits the toxin was absorbed much more readily from the upper small intestine than from the lower (ileal) region. Absorption from the stomach could be demonstrated in only 2 of 11 rabbits.

In rabbits, absorbed toxin appeared in high titre in thoracic duct lymph and terminal plasma frequently contained toxin. Of the total absorbed at least 50 per cent in many experiments, was collected in thoracic duct lymph. Cannulation of the intestinal lymph duct and continuous collection of lymph following oral ingestion of toxin by rats gave either complete or partial protection from toxaemia.

It is concluded that the lymphatics and not the portal blood transport absorbed toxin to the systemic circulation.

When toxin was fed by stomach tube to rats, rabbits and mice most of the administered dose was destroyed or in some other way prevented from being absorbed. Oral toxin was more effective in mice if given during or immediately following a normal feeding period after a 20–22 hr. fast.

Toxin given i.v. was only slowly removed from the circulation.

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A STUDY OF INFECTION IN ORGANISED TISSUE CULTURES

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ONE of the most striking phenomena of infection is the tissue or cell specificity involved in the interaction between host and parasite. This has usually been studied in the intact animal, which however is difficult to analyze. Goodpasture, Buddingh and their colleagues (Buddingh, 1952) made important advances by studying infection in the developing chick embryo and application of tissue culture procedures has increased our knowledge in this field. Many of these recent observations however have limited application to problems of specificity in infectious disease since the types of cells used, either dispersed cell culture or cell lines derived from tumours or mesoblastic tissues, have lost their normal anatomical relationships and no longer show characteristic differentiation. In the quest for a satisfactory host in which to study specificity uncomplicated by the influence of other organs or tissues, we have exploited a method originally devised by experimental embryologists (Fell, 1951) in which fragments or whole rudiments of embryonic tissue are cultivated *in vitro* in order to investigate the factors controlling development. This paper reports the cultivation for periods of 2 to 3 months of organised tissues, human and animal, embryonic and adult, and records preliminary results of the infection of some of them with agents known to infect such tissues in the natural host. Nasal, tracheal and bronchial mucosal tissues have been particularly studied as we have a special interest in viruses which infect the respiratory tract.

MATERIAL AND METHODS

Human embryos, which varied in length up to 10–12 cm. were received 3–12 hr. after removal and used immediately. Adult human tissue, mainly bronchial epithelium, was taken from operation specimens from cases of carcinoma, bronchiectasis and tuberculosis obtained immediately after excision and explanted at once or within 24 hr. after storage at 4°. Fragments of trachea from day-old chicks and the nasal mucosa covering the maxillo-turbinate of adult ferrets were used for studies of Newcastle disease and influenza virus respectively. The culture technique employed was essentially that described by Fell and Robinson (1929) in which the medium is contained in a small watch-glass enclosed in a Petri dish and surrounded by a ring of wet cotton wool. Two types of medium were used, either a semi-solid fowl plasma clot containing embryo extract from 12–14-day embryos diluted to 50 per cent with Gey's balanced salt solution and sufficient glucose to give a final concentration of 300–400 mg. per 100 ml. or a liquid medium obtained by grinding up plasma—embryo extract—glucose clots. In all cases, the tissue fragments were supported on the surface of the medium by small squares of rayon curtain material, prepared according to the technique devised by Shaffer (1956). The cultures were incubated at 37° without CO_2 and transferred with the supporting rayon to fresh medium every 5–7 days. During the cultivation period, observations were made with a low-power Zeiss binocular microscope through the top lid of the Petri dish; improved visualisation can be obtained by substituting an optical flat of glass, flamed before use, for the Petri dish lid. The material obtained by withdrawing a small

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drop of fluid from around the culture with a micro-pipette was also examined during the life of the cultures and attempts were also made to ascertain the relative numbers of dead and living cells by placing a few drops of concentrated trypan blue solution on the culture for 15 min. and after withdrawal, washing the culture gently with warm, balanced salt solution. This method was not always satisfactory since the mucus secretion from many of the cultures contained degenerate cells and itself tended to stain metachromatically. After varying periods of time, the cultures were fixed for histological examination in Zenker's fluid containing 3 per cent acetic acid or in Carnoy's fluid. A few cultures were fixed for ultra-thin microtomy by placing the culture with its rayon support in 2 per cent osmium tetroxide dissolved in veronal acetate buffer—ph-7·2–7·4. As with light microscopy procedures, the rayon is removed with acetone prior to embedding. Sections were cut on a Porter-Blum ultra-microtome.

RESULTS

Embryonic human trachea

Human respiratory tract tissue was extensively studied on account of a special interest in the virus of the common cold. Adequate amounts of tracheal tissue for 6–10 cultures were available from each of 5 embryos. The fragments, about 5 mm. square, consisting of mucosa and underlying cartilage were maintained for periods up to 2 months. Immediately after explantation, the epithelial surface, always kept uppermost, had a smooth velvety appearance. The openings of the mucous glands which penetrate the submucosa could be clearly seen. Within 3 days, epithelial cells and fibroblasts from the edges of the explant had migrated over the surfaces and into the rayon net. Ciliary action could be easily discerned by spot-lighting the culture from an angle; this oblique illumination revealed rapid pulsation of the thin layer of surface fluid, clearly visible at magnifications of $\times$ 25 and $\times$ 40. Although this fluid movement tended to disappear as the cultures sank into the clot, rhythmic fluid movement again became visible when they were transferred to fresh medium. Cultures of nasal mucosa obtained from 3 embryos were explanted in the same way except that in this case, the mucosa was stripped from the underlying cartilage. The appearance and behaviour of these cultures were similar to those of the tracheal cultures, ciliary movement could be readily identified, but the fragments tended to become flattened and spread over the rayon net, probably because of the removal of the cartilage. The mucus which was continuously secreted did not accumulate in any particular area. A histological section of a tracheal culture is shown in Fig. 2 and can be compared with uncultured normal trachea, Fig. 1.

Many of these cultures were used for studying the common cold virus, using passage material obtained from the Common Cold Research Unit, Harvard Hospital, Salisbury. No histological changes were found in the tissue cultures and an occasional equivocal result in a series of otherwise negative findings following inoculation into human volunteers was in line with earlier tissue culture experience in this problem. A number of tracheal explants were inoculated with various strains of chick-adapted influenza. Since it was not known how readily the virus would penetrate the layer of mucus and reach the cells, fairly large inocula were used. Although virus was recovered in some cases, no definitive lesions were demonstrated and it may well be that the strains had lost their pathogenicity for human tissues. This seems all the more likely since a recently isolated strain, which had been passed only twice in chick embryo, failed to produce symptomatic or serological evidence of influenza when inoculated into human volunteers (Isaacs and Roden, 1956).

Adult human bronchus

Adult human bronchial mucosa was studied late in the course of these experiments and is now under further investigation. In most cases the mucosa was stripped off the underlying cartilage and the fragments placed surface upwards on rayon net. Active ciliary movement was easily visible at the outset but after several transfers, the activity diminished, the epithelium at the edge became translucent and a cloudy haze of mucus collected round the cultures. The fine structure of these cultures was studied at higher resolutions and Fig. 5 and 6 show that the rather sparse cilia have the same basic structure described for cilia in general (Fawcett and Porter, 1954) and characteristic microvilli occur on mucous cells and between the cilia. The basement membrane is well defined with the basal cells applied directly to it.

Ferret nasal mucosa

The susceptibility of the nasal mucosa of the adult ferret to influenza virus is one of the most striking examples of tissue-parasite specificity and it seemed of special importance to study the behaviour *in vitro* of adult tissue of this type, especially since comparatively little is known concerning the cultural requirements of fully differentiated adult tissue maintained as organized fragments. It was of additional interest also to determine whether cells from an immune host in their normal anatomical relationships would lose their resistance, as they do in the usual type of unorganized tissue culture (Andrewes, 1930) or when explanted on to chick chorio-allantoic membrane. The mucosa from the maxilloturbinate of normal ferrets was cut into fragments in a solution containing 100 units per ml. each of streptomycin and penicillin. Orientation of the explants was difficult but in most cases it was possible to place the epithelial surface uppermost. The cultures were maintained *in vitro* for relatively short periods—up to 14 days. When inoculated with the W.S. strain of influenza virus, the surface cells were rapidly destroyed, and the process could be followed by removing small drops of fluid from around the culture and examining these fresh by phase contrast microscopy. From the uninfected control cultures, only occasional desquamated cells were obtained, whereas in the infected cultures, ciliated and mucus-secreting cells were commonly present in the culture. The mucosa from "immune" (convalescent) ferrets explanted in the same way was just as susceptible as that from normal ferrets within 2 days of explantation.

Chick trachea

Short trough-shaped segments of trachea from 1-day-old chicks were cultivated with the mucosa side upwards and the outer surface of the cartilage ring lying directly on the rayon net. Although it was not possible to observe ciliary action in these cultures, the mucus which was secreted into the trough was continuously dumped at one end and this motion could be studied by placing a small drop of India ink at the end from which the flow began and observing the passage of particles to the other end. Such cultures were inoculated directly with 2 strains of Newcastle disease virus of differing virulence for embryonic and adult fowl tissues. The virulent strain was known to be pathogenic for both embryonic and adult tissues, the avirulent pathogenic for embryonic tissues only. With the virulent strain, patchy epithelial necrosis took place and there was some destruction

of underlying fibroblasts, but with the avirulent, only epithelial hyperplasia was found; in neither case were the lesions extensive. Whether or not the thin sheet of mucus covering the cultures modified the course of infection was not determined, but since virus was recovered following minimal inoculations (the infective titre rose from 10^{-5} to 10^{-8}) it is likely that multiplication did occur under these conditions.

Embryonic skin

The behaviour of embryonic avian skin *in vitro* has been described by Fell and Mellanby (1953) who utilised cultures of this type for studying the effect of vitamin A, and who found that an excess of vitamin A produced a transformation of the ectodermal cells into a mucus-secreting and often ciliated epithelium resembling nasal mucosa. Normal control cultures underwent a characteristic differentiation with keratinization and some degree of development of the feather germs. Following explantation, embryonic human skin showed growth and differentiation of the epithelial layers comparable to that observed by Fell and Mellanby in their control cultures, with keratinization of the superficial cell layers and considerable development of hair follicles. Some of the cultures were infected with vaccinia and herpes simplex viruses. The growth of the vaccinia was so extensive that in 2 days most of the epithelial cells showed cytoplasmic inclusion bodies. The herpetic infection resulted in the formation of a tiny fluid-distended vesicle mimicking the lesion found *in vivo* with typical intranuclear bodies in the epithelial cells (Fig. 7 and 8).

Embryonic liver

The growth characters of embryonic chick and human liver were investigated by the techniques described and contrary to expectations, the solid liver fragments were found to survive better when supported with rayon on a semi-solid plasma clot than when floating on silicone-treated rayon on fluid media. In all cases however, a considerable degree of central necrosis took place with fibroblastic replacement. In some cultures also there was active phagocytosis by Küpffer cells of erythrocytes and erythroblasts contained within the fragment, with formation

EXPLANATION OF PLATES

FIG. 1.—Section of embryonic human trachea before explantation *in vitro*. Gomori's trichrome stain. × 150.

FIG. 2.—Section of embryonic human trachea after 4 weeks' cultivation *in vitro*. Note the preservation of normal micro-anatomical structure. Gomori's trichrome stain. × 220.

FIG. 3.—Section of adult human bronchus, cultivated for one month, showing patent vascular channels below the mucosa and a layer of mucus covering the surface. P.A.S. and haematoxylin. × 150.

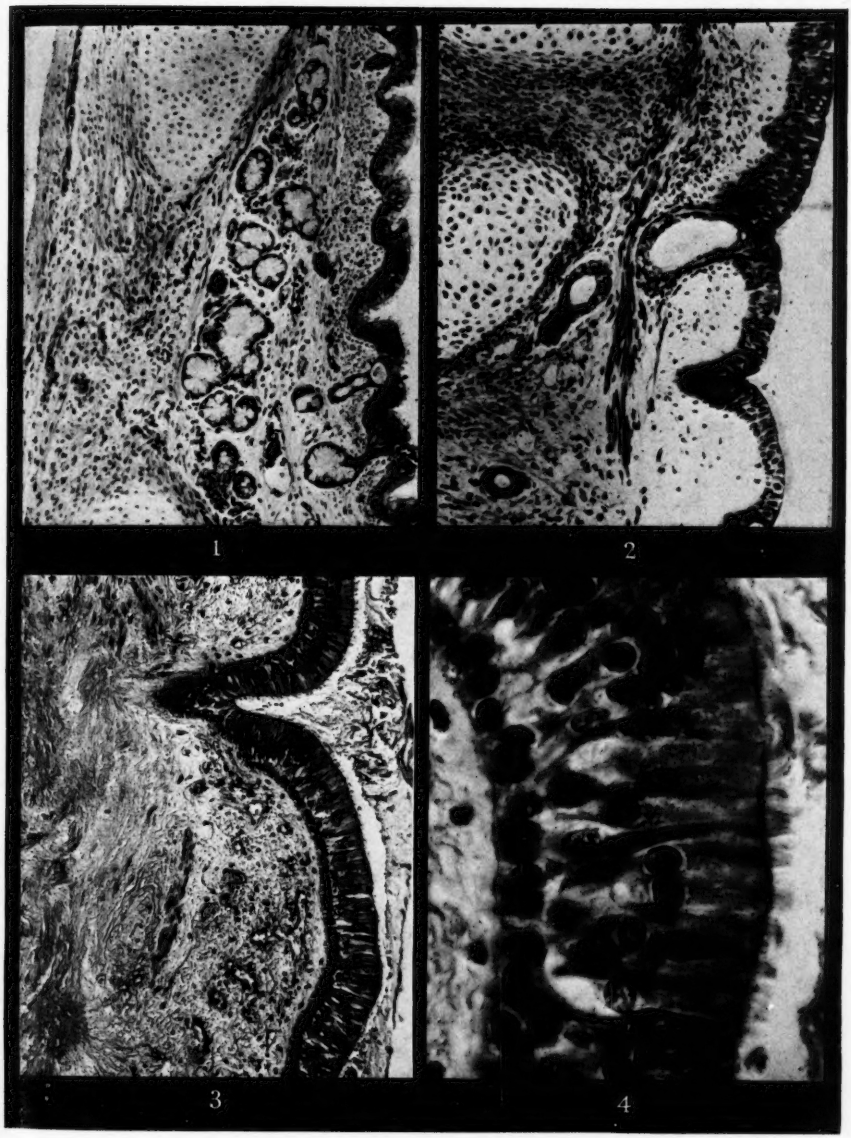
FIG. 4.—The mucosa from Fig. 3 showing the regular arrangment of the ciliated cells, some mucus-secreting cells and a well-defined basal layer. P.A.S. and haematoxylin. × 750.

FIG. 5.—Electron micrograph of section of cilia from surface of culture similar to Fig. 4. A group of microvilli is seen on the left of the field. × 20,000.

FIG. 6.—Electron micrograph of section from another portion of the same culture showing secretion of mucus in the form of small droplets within and outside the cell surface. × 33,200.

FIG. 7.—Section of a vesicle in a culture of human embryonic skin, 14 days after explantation and 3 days after infection with herpes simplex virus. P.A.S. and haematoxylin. × 88.

FIG. 8.—Same section showing characteristic intranuclear inclusions of herpes simplex. P.A.S. and haematoxylin. × 780.



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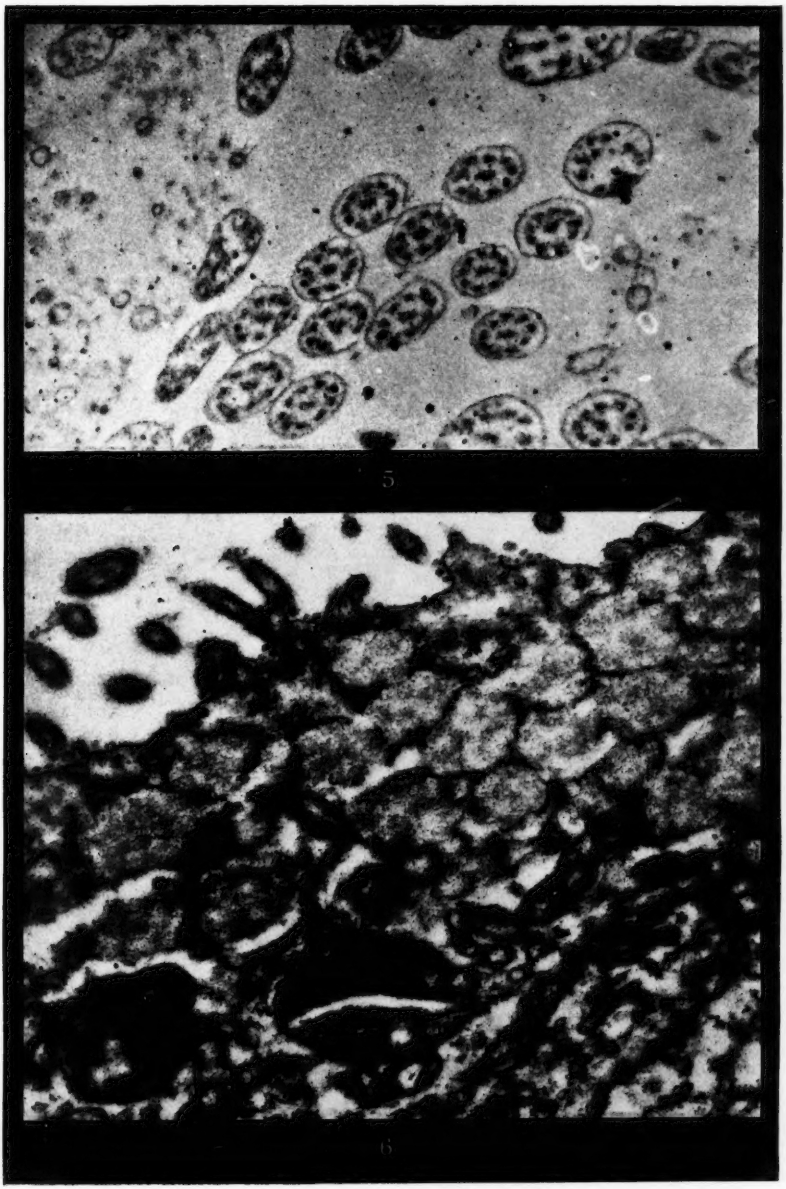

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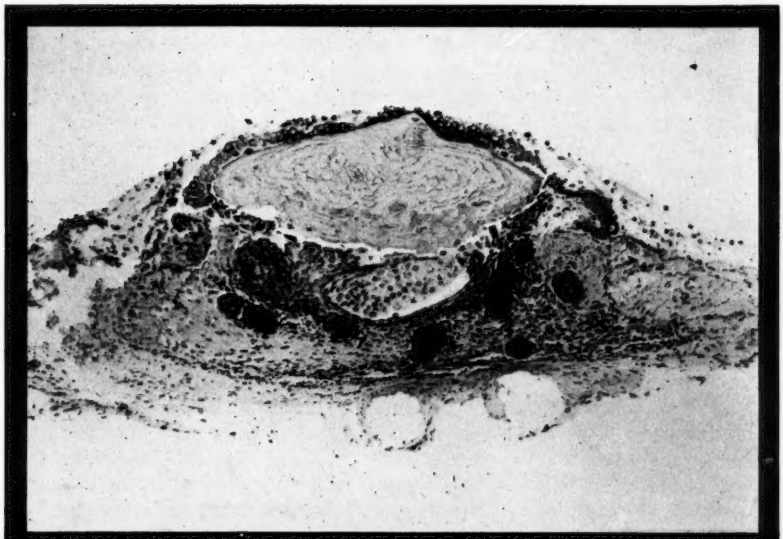

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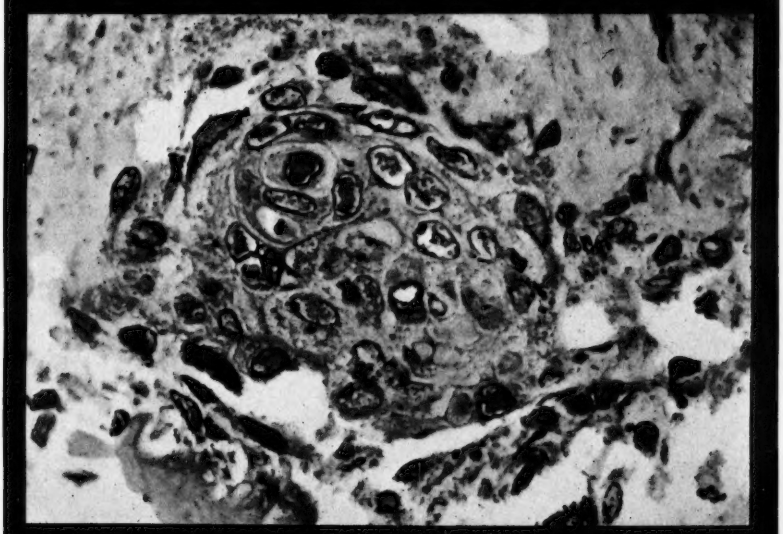

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of breakdown products of haemoglobin including haematoidin crystals. In order to investigate the possibilities of this tissue culture method for another type of host-parasite relationship, several cultures previously treated with penicillin and streptomycin were infected with large doses of *Plasmodium vivax* sporozoites from infected mosquitoes by Mr. P. G. Shute, of the Malaria Reference Laboratory, Horton Hospital, Epsom, using one mosquito for each fragment. The cultures were maintained at temperatures of 33° and 37° and observed for 6–8 days. Although several kinds of suggestive developing exoerythrocyte forms were found within liver cells, serial sections of the cultures did not reveal a sufficient number of parasites to justify any correlation with the heavy inocula. Cultures of embryonic liver were also used in attempts to cultivate the virus of human and chick hepatitis and the results have been reported briefly elsewhere (Bang, 1957).

DISCUSSION

Although one of the aims of this study, the growth *in vitro* of the common cold virus in organised cultures of respiratory epithelium was not attained, the few experiments here reported demonstrate clearly that certain specific agents, such as influenza virus, vaccinia and herpes simplex virus can produce on ferret nasal mucosa and human ectoderm respectively cellular reactions comparable to those which occur in natural infections or in an appropriate experimental animal. But before attempting to assess the usefulness of organized tissue cultures in infectious processes, it is necessary to consider the limitations as well as the possibilities of the technique. At the present time, comparatively little is known regarding the nutritional requirements of such cultures, the action of vitamins, the influence of tissue age in maintaining viability and the effect of different substrates. Fowl plama is very generally used since in the experience of most workers it provides a more suitable clot than mammalian plasma but it is not a natural substrate for many of the tissues in this study. Moreover, although organized cultures maintain an astonishing micro-anatomical integrity, only certain types of physiological activity such as ciliary activity and mucus formation in the respiratory tract tissues could be assessed and there is no means of determining the effect on function of phagocytosis within the culture, cellular hyperplasia at the margins or central necrosis. Despite all these predictable difficulties, however, it seems certain that the study of infection in organized tissues both embryonic and adult outside the host offers great promise for the analysis of problems of specificity, and in a recent paper, Wolff (1956) calls attention to the application of similar methods for the study of the spread of tumour cells in organized tissue explants.

SUMMARY

Small fragments of human embryonic trachea and nasal mucosa and adult human bronchial mucosa were grown as organised cultures *in vitro* using the watch glass-rayon net method. The two characteristic functions of ciliation and mucus secretion were maintained for at least two months. The human embryonic cultures were not susceptible to infection with strains of chick-adapted influenza virus.

Adult ferret mucosa was similarly grown. The ciliated and mucus cells were destroyed by influenza virus but the deep mucosal glands and in some cases the

cubical basal cells were preserved. Similar tissue taken from ferrets immune to influenza virus was equally susceptible *in vitro*.

Pieces of chick trachea maintained their functional integrity *in vitro*, secreting mucus which was carried by ciliary movement to one end of the trough-shaped fragments. These cultures were infected with two strains of Newcastle disease virus of differing virulence but only minimal lesions were apparent.

Human embryonic skin cultures were infected with vaccinia and herpes viruses and characteristic lesions were produced.

Human and embryonic liver fragments were grown for several weeks in organised culture. Tissue survival was mainly at the rim of the explant and central necrosis was common. Infection of the human liver cultures with sporozoites of *Plasmodium vivax* produced equivocal results.

Much of this work could not have been carried out without the co-operation of surgical colleagues in the London area who supplied human embryos when these were available. We wish to acknowledge the interest and encouragement of Dr. C. H. Andrewes, during the course of the work. We are specially indebted to Dr. A. E. Karrer, Johns Hopkins University, Baltimore, for Fig. 6, and to Dr. R. C. Valentine, National Institute for Medical Research, Mill Hill, London, N.W.7 for Fig. 5. The other photomicrographs were prepared by Mr. M. Young, National Institute for Medical Research, Mill Hill, London, N.W.7.

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THE PRODUCTION OF IMMUNOLOGICAL UNRESPONSIVENESS BY THE INTRAVENOUS INJECTION OF BOVINE SERUM ALBUMIN INTO THE CHICK EMBRYO*

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MANY investigators have reported the successful reduction of the immunological response to soluble protein antigens (Hanan and Oyama, 1954; Dixon and Maurer, 1955; Cinader and Dubert, 1955; Smith and Bridges, 1956). These workers were able to bring about this effect by injecting large quantities of these antigens into foetal and new-born rabbits. Recently Wolfe *et al.* (1957) reported that by injecting newly hatched chicks (8–50 hr. of age) intraperitoneally with bovine serum albumin (BSA) antibody production to the homologous antigen was lessened. The duration and degree of this reduced responsiveness seemed to depend upon the amount of the original injection.

Simonsen (1956) was able to produce immunological unresponsiveness by injecting human erythrocytes directly into the chick embryo. He observed that this effect depended upon the day the initial injection was made and the interval between this injection and the challenging injection. Chick embryos injected prior to the 15th day of incubation failed to show " partial immunological tolerance ". Animals injected between the 17–19th days gave the best results. Billingham, Brent and Medawar (1956) were able to prolong the life of tissue transplants by injecting Rhode Island Red (RIR) blood cells into 10–11-day White Leghorn (WL) chick embryos. The injection of human serum albumin into the allantoic cavity of chick embryos will produce partial tolerance (Stevens, Pietryk and Ciminera, 1958).

Hašek (1956) was unable to bring about a suppression of agglutinins when he intravenously injected chick embryos at 13 days with turkey, guinea-fowl or duck blood. The injection of goose blood into 15-day chick embryos led to a suppression of agglutinins but injections of duck embryos with chicken blood led to no suppression.

Cohn (1957) using several different antigens, reported that intravenous injection of 14-day chick embryos failed to inhibit the ability of these animals to respond to the homologous antigen when challenged at 10–12 weeks of age.

This investigation is concerned with immunological responsiveness of chickens as affected by injecting bovine serum albumin intravenously into the developing chick embryo.

MATERIALS AND METHODS

Arbor Acre White Rock chickens, approximately 50 per cent males, were used in these experiments. All embryos except 6 were injected on the 15th day of incubation; six were injected at 18 days. The experimental birds in Experiment I received 37·5 mg. (in 0·2

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ml.) bovine serum albumin (BSA) supplied by Pentex, Inc., and those in Experiment II, 46·8 mg. (in 0·25 ml.) BSA. All injections were made into the chorio-allantoic vein of the embryos. These embryos were hatched and maintained in our laboratory until they were challenged and subsequently sacrificed.

Groups of previously embryonically injected and uninjected animals were inoculated intravenously with 40 mg. of BSA per kg. body weight (KBW) at 6 and 12 weeks of age. On the 4th and 6th days following the injection a small amount of blood was removed from the wing vein in both groups and the sera were tested for the presence of antigen or antibody. The presence of antigen was determined by the flocculation technique employing a strong homologous antiserum. On the 8th day all animals were bled by cardiac puncture and sacrificed. The serum was collected in the usual manner and allowed to stand for 3–4 weeks in the refrigerator at 4° for stabilization (Gengozian and Wolfe, 1957).

Where antibody was present the amount of antibody nitrogen was determined by the method developed by Heidelberger, *et al.* (1933). The reaction mixture of 1·0 ml. contained 0·25 ml. of antiserum, 0·25 ml. of the antigen solution in 8 per cent saline and 0·5 ml. of 11·5 per cent NaCl. This brought the salt concentration of the reacting mixture up to 8 per cent which was shown by Goodman, *et al.* (1951) to be optimal for the chicken system. The antigen concentrations employed were at 1 μg. N intervals and were in the region of equivalence. These mixtures, run in duplicate, were incubated for 3 hours at 37° and then kept in the cold (4°) for approximately 18 hr. They were then centrifuged at 4° for 30 min. at 3000 r.p.m. The supernatant fluids were saved and tested for the presence of antigen and antibody. The precipitates were washed with 5·0 ml. of cold 8 per cent NaCl and recentrifuged. The nitrogen determinations of the precipitate were made by the method of Johnson (1940). The antibody nitrogen per ml. of serum was determined by using the region of slight antigen excess. When necessary, because of high antibody content, the antiserum was diluted. The determinations were then calculated using the formula devised by Gengozian and Wolfe (1956) for diluted chicken antisera. It was not possible to measure antibody contents accurately below 25 μg. antibody N/ml. All sera tested that gave a positive flocculation test but contained less than 25 μg. N/ml. were arbitrarily recorded as 15 μg. N/ml. and this figure was used in the calculations.

All sera that were negative by the flocculation test (in 8 per cent saline) were retested using the interfacial test at a salt concentration of 2 per cent.

RESULTS

In Experiment I, 40 6-weeks-old Arbor Acre White Rocks were injected intravenously with 40 mg. KBW of BSA; the data for these are recorded in the table. Twenty of these birds received 37·5 mg. (in 0·2 ml.) BSA as embryos on the 15th day of incubation. Approximately 3·0 ml. of blood were drawn from several of the control and experimental animals on the 4th and 6th days. These sera were tested for the presence of antigen and antibody. The results of these tests indicated that the time of antigen disappearance and antibody appearance in the circulation was about the same in both groups. Antigen disappeared from the circulation sometime between the 4th and 6th day with antibody being observed in those animals producing antibody by the 6th day. The results of the 6-weeks-old group of animals indicate that it is possible to produce partial immunological unresponsiveness by this method. The average mean titre of the experimental animals was 108 ± 19 μg. antibody nitrogen per ml. (N/ml.) as compared with the control values which averaged 197 ± 33 μg.N/ml. of serum. When these two groups of animals were compared statistically using the Z-score test for significance the P-value was 0·0102 (Mode, 1951).

Similarly at 12 weeks of age another group of 40 animals was injected with 40 mg. BSA per KBW. This group consisted of an equal number of control and experimental animals. Eight days after the injection the experimental animals had antibody nitrogen values similar to those of the control birds. The mean

titre of the animals was 336 ± 94 μg.N/ml. in contrast to 389 ± 96 μg. N/ml. for the control animals. The two groups compared statistically by the *Z*-score test showed no significant differences ($P = 0{\cdot}3483$).

In Experiment II all experimental embryos received 46·8 mg. (in 0·25 ml.) of BSA. At 6 weeks of age 13 birds injected at 15 days and 6 injected on the 18th day of embryonic development along with 19 control animals were injected with 40 mg./KBW of BSA. As in the previous experiment the time of antigen disappearance and antibody appearance was determined. The antigen disappearance in the experimental groups was the same whether the animals produced antibody or not. It was possible by the use of 46·8 mg. of BSA injected into the chick embryo to produce almost complete unresponsiveness in 6-weeks-old animals. Of the total of 19 experimental animals, 14 failed to produce antibody (10 were injected as 15-day and 4 as 18-day embryos). All of the negative animals were tested by both the interfacial and flocculation tests. In this group all of the control animals produced antibody. The mean titres for the experimental animals were 11 ± 6 μg. N/ml. for the 15-day group and 25 ± 17 μg. N/ml. for the 18-day animals. This was in contrast to 152 ± 18 μg. N/ml. for the control group. When the two experimental groups were compared statistically with the controls, using the *Z*-score test for significance, they both had a *P*-value of $< 0{\cdot}0001$ (see the table).

An additional group which received 46·8 mg. BSA on the 15th day of embryonation was challenged at 12 weeks of age. Here, as in the previous experiment, the control and experimental groups responded in a like manner. The mean titre was 304 ± 68 μg. N/ml. and that for the control animals 317 ± 74 μg. N/ml.

A number of chickens were bled as late as 14 days after the challenging injection. There was no indication that a delayed response occurred.

TABLE.—*Bovine Serum Albumin Injected into Chick Embryos and the Statistical Analyses of the Antibody Response of these Animals*

		Six weeks of age				Twelve weeks of age			
Exp. No.	Preliminary treatment	No. of birds	Range (μg. N/ml.)	Mean ±S.E.	Statistical evaluation*	No. of birds	Range (μg. N/ml.)	Mean ±S.E.	Statistical evaluation
1	37·5 (in 0·2 ml.) on 15th day incubation	20	0†–260	108±19	$Z=2{\cdot}32$ $P=0{\cdot}0102$	20	0§–1068	336±94	$Z=0{\cdot}39$ $P=0{\cdot}3483$
	None (controls)	20	0‡–612	197±33		20	64–792	389±96	
2	48·6 mg. (in 0·25 ml.) on 15th day	13	0‖–64	11±6	$Z=7{\cdot}38$ $P=0{\cdot}0001$	13	15–704	304±68	$Z=0{\cdot}13$ $P=0{\cdot}4483$
	48·6 mg. (in 0·25 ml.) on 18th day incubation¶	6	0**–96	25±17	$Z=5{\cdot}14$ $P=0{\cdot}0001$				
	None (controls)	19	32–304	152±18		16	0–1089	317±74	

* Using *Z*-score test for significance and the single tailed test for probability.
† Two animals failed to produce antibody.
‡ Two animals failed to produce antibody.
§ One animal failed to produce antibody.
‖ Ten animals failed to produce antibody.
¶ Only 6 chickens survived to be challenged.
** Four animals failed to produce antibody.

DISCUSSION

The results reported in this investigation compare favourably with our previous report of the effects of injected, newly hatched chicks (Wolfe *et al.*, 1957). We were unable to bring about complete unresponsiveness with the procedures we employed. In the two experiments reported here, the unresponsiveness produced was transitory. Experimental group I demonstrated partial unresponsiveness while in those in Experiment II this inhibition was almost complete at 6 weeks of age. However, by the 12th week this inability to respond was lost in both experimental groups and they responded as well as the control groups. It appears that the injection of 15-days-old chick embryos with the dosage used in these studies does not produce any greater degree of unresponsiveness or prolong the duration of this effect than a similar injection given soon after hatching (Wolfe *et al.*, 1957). In fact the duration is shorter since when injecting with large doses at post-hatching the effect lasted 12–22 weeks. These results with embryos compare with the results obtained by Simonsen (1956) and Stevens *et al* (1958). They, too, were unable to bring about complete unresponsiveness and Simonsen showed that by the second month this inability to respond had disappeared.

However, these results differ from those reported by Cohn (1957). Certain reasons may account for Cohn's inability to alter the immune mechanism. The amount of antigen he injected into the chick embryo was small. In addition, he waited until the 10–12th week to challenge his animals and in view of the studies reported here and those of Simonsen (1956), this seems to be too long an interval to wait to test for immunological unresponsiveness. The day of injection may also be of significance, because Simonsen (1956) was unable to produce any effect earlier than the 15th day and Cohn chose the 14th day for his injections. Further work is needed on determining the time of the inital injection which will produce the maximum effect in chickens.

The rate of antigen disappearance in the partially or completely unresponsive animals was similar to the control animals. Antigen was present in all animals on the 4th day bleeding but was absent by the 6th day. No reason can be given for the rapid rate of disappearance in the experimental animals. Preliminary tests do not indicate the presence of incomplete antibody. This is not in agreement with other workers who worked with rabbits (Hanan and Oyama, 1954; Cinader and Dubert, 1955; Smith and Bridges, 1956). They all reported a slower disappearance of antigen from the circulation in their non-responding animals. It should be noted that these other workers used mammals.

SUMMARY

A reduced immunological responsiveness at 6 weeks of age was produced in chickens when 15-days-old embryos were intravenously injected with 37·5 mg. BSA and almost a complete inhibition when the dosage was 46·8 mg. BSA. This effect disappeared by the 12th week.

The time of antigen disappearance from the circulation of the experimental groups was similar to the control group, whether or not the experimental animals produced antibody.

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THE EFFECT OF DOSAGE AND TIME OF INJECTION OF A SOLUBLE ANTIGEN ON THE PRODUCTION OF IMMUNOLOGICAL UNRESPONSIVENESS IN CHICKENS*

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In order to produce immunological unresponsiveness to a particular substance it is necessary to expose an animal with the substance prior to the " maturation of the faculty of immune response " (Billingham, Brent and Medawar, 1956). The time such maturation takes place varies with the animal species and also with the type of antigen to which it is exposed. Billingham *et al.* (1956) were able to produce a high degree of " tolerance " to homografts by injecting blood cell suspensions into newly hatched chicks. Wolfe, Tempelis, Mueller and Reibel (1957) were able to produce a lowered precipitin production when newly hatched chicks were given massive doses of a soluble protein antigen. Hanan and Oyama (1954), Cinader and Dubert (1955, 1956), Dixon and Maurer (1955) and Smith and Bridges (1956) found the new-born rabbit a good animal in which to produce immunological unresponsiveness. These workers injected soluble protein antigens and observed a high degree of unresponsiveness when the animals were challenged at maturity. Smith and Bridges also were able to bring about this inability to respond in 50 per cent of the rabbits inoculated at 15 days of age. Other workers demonstrated " tolerance " to skin grafts in rats injected with homologous cells at 2 weeks of age (Woodruffe and Simpson, 1955).

It is usually necessary to inject a fairly large amount of protein antigen initially in order to produce immunological unresponsiveness. Cinader and Dubert (1955) succeeded by injecting new-born rabbits with a total of 23 mg. of human albumin given over a period of 19 days, while Smith and Bridges (1956) produced the effect with 20 mg. bovine serum albumin. The latter experiments indicate that in rabbits these quantities of antigen produce inhibition for at least 8 months. In chickens, 47 mg. given soon after hatching depressed the antibody production to the homologous antigen at 6 weeks of age but by the 12th week the animals regained their ability to respond as well as normal animals of the same age (Wolfe *et al.*, 1957).

This investigation represents an attempt to ascertain the maximum age and the minimal amount of antigen that must be injected for the production of immunological unresponsiveness in the chicken.

MATERIALS AND METHODS

Arbor Acre White Rocks chicks consisting of approximately 97 per cent males were purchased from a local hatchery. The animals in Experiment I were injected during the 1st day after hatching with varying amounts of bovine serum albumin (BSA) supplied by

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Pentex, Inc. In Experiment II the chicks were injected at either 1, 4, 8 or 12 days post-hatching with comparable amounts of the antigen based on their body weights. Details of the dosage received by each group will be described in " Results ". All injected birds and a sufficient number of uninjected animals were maintained in our laboratory until they were challenged and subsequently sacrificed. A total of 377 animals were used in these experiments.

Groups of animals in Experiment I were injected intravenously with 40 mg. per kg. body weight (KBW) at 6, 12 and 22 weeks and those in Experiment II at 6, 8 and 12 weeks. The procedures used for sera collection and the methods followed for determination of circulating antigen and antibody are described in an earlier publication (Tempelis, Wolfe and Mueller, 1958).

RESULTS

In Experiment I initial injections given on the first day after hatching included three different doses of BSA. Animals in group I received 3·76 mg. (in 0·1 ml.); group II were injected with 22·5 mg. (in 0·12 ml.) and group III received 135 mg. (in 0·72 ml.). The data for these are found in the table. At 6 weeks of age 41 experimental animals consisting of 15 in group I, 14 in group II and 12 in group III along with 14 control animals (group IV) were challenged with 40 mg. BSA per KBW. The results indicate that at 6 weeks of age, the group receiving the smallest initial dosage (3·76 mg.) responded by producing antibody titres similar to the control group. The 2 other experimental groups produced antibody in a quantity significantly lower than the control animals. The titre of the group which received 22·5 mg. BSA was 61 ± 16 μg. N/ml. and that of the group receiving 135 mg. BSA was 33 ± 15 μg. N/ml. These were in contrast to the control group which had a mean titre of 164 ± 19 μg. N/ml. When these 2 experimental groups were compared statistically to the control birds by the Z-score test for significance the P-value for each group was less than 0·0001 (Mode, 1951).

At 12 weeks of age additional animals from all 3 groups indicated that the experimental and control groups respond in a like manner. At 22 weeks of age groups I and II which received the smaller amount of antigen at post-hatching responded as well as the controls. On the other hand, group III which received the largest initial dose showed a significant reduction in response. The mean titre of this experimental group (11 animals) was 306 ± 47 in contrast to 596 ± 113 for the control group (21 animals). When these 2 groups were compared by the Z-score test of significance the P-value was 0·009. We cannot explain why this group (III) showed a significant reduction in responsiveness at 22 weeks but not at 12 weeks.

The animals in Experiment II were divided up into 5 groups, one of which was the controls (V). The data for these are recorded in the table. Group I received 112 mg. (in 0·6 ml.) of BSA during the first day post-hatching. The mean weight for this group was 43 g. Groups II, III and IV were injected at days 4, 8 and 12 respectively. The amount of BSA injected into each was based on its weight and was comparable to that given animals of group I (112 mg. per 43 g. chick).

At 6 weeks of age all 4 experimental groups consisting of 13 animals each and 15 control birds received 40 mg./KBW of BSA intravenously. The mean titres for the experimental groups were: day 1, 55 ± 17; day 4, 41 ± 17; day 8, 69 ± 16, and on day 12, 62 ± 18 μg. N/ml. Each experimental group was compared to the control animals by the Z-score test. The P-value in each case was less than 0·0001.

TABLE.—*Antibody Response of Chickens Pre-treated with Various Concentrations of Bovine Serum Albumin at Hatching and on Different Days following Hatching*

Group No.	Preliminary treatment	Ab N μg./ml. range	Mean ±S.E. p value	Ab N μg./ml. range	Mean ±S.E. p value	Ab N μg./ml. range	Mean ±S.E. p value	Ab N μg./ml. range	Mean ±S.E. p value
		Six weeks of age		Eight weeks of age		Twelve weeks of age		Twenty-two weeks of age	
		Experiment No. 1							
I	3·76 mg. in 0·1 ml. on Day 1	0–292 (15)*	132±24 $p=0·15$	—	—	28–496 (14)*	270±36 $p=0·48$	168–1140 (15)*	424±68 $p=0·097$
II	22·5 mg. in 0·12 ml. on Day 1	0–168 (14)	61±16 $p=<0·0001$	—	—	15–642 (14)	204±51 $p=0·14$	220–940 (12)	454±72 $p=0·14$
III	135 mg. in 0·72 ml. on Day 1	0–164 (12)	33±15 $p=<0·0001$	—	—	72–432 (12)	248±33 $p=0·33$	152–647 (11)	306±47 $p=0·009$
IV	None (Controls)	48–304 (14)	164±19	—	—	124–582 (14)	272±41	36–2024 (21)	596±113
		Experiment No. 2							
I	112 mg. in 0·6 ml. on Day 1	0–220 (13)	55±17 $p=<0·0001$	15–232 (13)*	125±19 $p=<0·0001$	0–1408† (18)	264±226 $p=0·25$		
II	Based on weight‡ (Day 4)	0–184 (13)	41±17 $p=<0·0001$	0–180 (13)	71±14 $p=<0·0001$	208–692 (15)	299±32 $p=0·33$		
III	Based on weight‡ (Day 8)	0–176 (13)	69±16 $p=<0·0001$	0–184 (13)	80±17 $p=<0·0001$	140–1008 (12)	288±68 $p=0·33$		
IV	Based on weight‡ (Day 12)	15–224 (13)	62±18 $p=<0·0001$	36–324 (13)	164±22 $p=<0·0001$	88–1600 (15)	349±96 p§$=0·81$		
V	None (Controls)	15–472 (15)	200±29	144–472 (15)	314±30	15–672 (15)	323±47		

* Number in parentheses is number of animals.
† If 1408 value is left out p value = 0·009 ; see text for explanation.
‡ Amount of antigen injected based on weight and equivalent to 112 mg. BSA per 1-day chick that had average weight of 43 g.
§ Based on 2-tailed P-test ; all others on 1-tailed P-test.

Additional animals from each of the 5 groups were injected at 8 weeks of age. As with the 6-weeks-old animals, all four experimental groups demonstrated a reduced response. The experimental groups when compared to the control group by means of the Z-score test all had a P-value less than 0·0001.

The remaining animals treated as previously described were challenged at 12 weeks of age. The antibody content of the 4 experimental groups was not significantly different from the control group. In regard to group I of this age group which contained 18 birds it should be noted that one animal had an antibody content of 1408 μg. N per ml. serum and the next highest value was 384 μg. N per ml. If this serum was not included in the calculations then there would be a significant difference ($P = 0{\cdot}009$) between this group and the control group.

The sera of many of these animals (experimentals and controls) were tested for the presence of circulating antigen and antibody on the 4th and 6th days following the challenging injection. The results of these tests indicated that the disappearance of antigen and appearance of antibody were similar in all animals. Antigen was present in all animals on the fourth day but absent on the 6th day with antibody appearing by the 6th day in those animals producing antibody. We also tested the sera of a number of birds (this also was done in a previous study, Wolfe, *et al.*, 1957) up to 15 days after the challenging injection. In no case did we find a delay in antibody production resulting from the post-hatching inoculation.

In these experiments, of the 377 animals used 94 were controls and 283 experimentals. All control animals gave detectable antibody on the 8th day following the challenging injection but 18 of the experimental ones failed to do so. Fourteen of the experimental birds that failed to respond were in the group that were challenged at 6 weeks of age, 2 were challenged at 8 weeks and 2 at 12 weeks.

DISCUSSION

It is apparent from these studies that 1- to 12-days-old chicks injected intraperitoneally with relatively large doses of bovine serum albumin will show a depression in their antibody response to the homologous antigen when challenged at 6 or 8 weeks of age. Smith and Bridges (1956) observed that 50 per cent of their rabbits injected at 15 days of age failed to produce antibodies when challenged at 4 months. It has been suggested that the failure of response reflects alteration of an immune mechanism which is immature in the neonatal mammal or bird. This immaturity was demonstrated in chickens by Wolfe and Dilks (1948) who found that 10 out of 29 birds injected with beef serum at 2 weeks of age failed to produce precipitins and the rest produced low titre antisera.

Billingham *et al.* (1956) found that a 2-weeks-old chick will reject tissue homografts from 9–20 days after the transplant. If a comparison is made between these results and the results secured after injection of a soluble antigen it might be deduced that either the mechanisms involved in precipitin production and sloughing of tissue grafts are not similar or that the skin graft response is much more sensitive to a low order of immune response. In other words, the 2-weeks-old chick is immunologically immature in regard to precipitin production but immunologically mature in regard to its ability to slough tissue grafts.

The results reported in this paper show that it is necessary to give large doses of bovine serum albumin to chicks post-hatching in order to produce an

unresponsiveness at a later age. We were unable to produce a lowered antibody response in 6-weeks-old chicks with a post-hatching dose that was equivalent to 87·0 mg. protein per kg. body weight, but a dosage of 523·0 mg. was very effective. A point of interest in regard to the lower dosage is that not only did it fail to produce an inhibitory effect on antibody response but also, as far as we could tell, it failed to sensitize the birds for a secondary type of response. If 6-weeks-old or older chickens were given this amount of antigen intravenously they would produce high titre antisera, and when re-injected after 1 to 2 months would respond with a secondary type response.

We were unable to determine the maximum length of the post-hatching period in which an unresponsiveness could be produced, though doubtless such a period can be established. When chicks were injected at 1, 2, 4, 8 and 12 days post-hatching and challenged at 6 or 8 weeks their antibody response was significantly lower than in controls of the same age. However, there was a difference in the length of the unresponsive period of the chicks that were injected with larger doses (based on body weight) of antigen. Only those injected on the first day showed a statistically significant reduction in antibody production when challenged at 12 weeks of age.

It is difficult with the evidence available to discuss what mechanism or mechanisms are responsible for a reduction in immune responsiveness to a soluble antigen in the chicken. The difficulty is enhanced when we try to compare the phenomenon with what occurs in the rabbit under similar conditions. In the latter animal the unresponsiveness seems to be permanent, while in the chicken it is transitory.

Campbell (1957), proposing a theory for antibody formation, postulated that " ... antigenicity is often, and perhaps always, intimately associated with incomplete intracellular fragmentation of material." He further states, " On the other hand, the attachment of a large intact foreign molecule to RNA might inhibit completely the participation of RNA in protein synthesis." We assume this would mean antibody synthesis. Since such large quantities of antigen were injected by us we thought it possible that " quantities " of this material were still in the animals' tissue and prevented antibody synthesis. We, therefore, injected a number of 3- to 4-weeks-old chicks, that had been injected within 24 hr. after hatching, with large quantities of a potent antiserum in order to " neutralize " any persistent antigen. This group of chickens, when challenged at 6 weeks, showed the same degree of unresponsiveness as the ones that were not given any antiserum. This experimental approach may, however, deserve further attention, perhaps with even larger doses of antiserum.

SUMMARY

A reduction of the chicken's immunological response to BSA can be produced with the injection of 22·5 mg. of BSA on the day of hatching.

A very large dose of BSA (135 mg.) given on the day of hatching will not bring about a complete or permanent suppression of the antibody response.

Injections of a large quantity of BSA in chicks up to 12 days of age produces a significant suppression of antibody formation when the birds are challenged at 6 and 8 weeks old.

The immunological unresponsiveness is transitory and its degree depends upon the size of the initial dose and the interval between this inoculation and the challenging injection.

Reduction in the unresponsiveness could not be changed by injecting large quantities of homologous antisera.

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SURVEY OF PAPERS

GREEN inoculated rabbits with splenic material from rabbits that had had courses of nitrogen mustard followed by simple intravenous inoculations of *Salmonella typhi* H cells at various times after the completion of the nitrogen mustard course. He shows that the recipient's response to the antigen is of the primary type if the splenic material comes from animals that either have not had a nitrogen mustard course or had finished the course 72 hr. or 96 hr. before the *Salm. typhi* inoculation. Animals that received material from donors who were inoculated with *Salm. typhi* 24 hr. or 48 hr. after the end of nitrogen mustard courses gave a secondary type of response (p. 213).

OLITZKY and SULITZEANU have applied a variety of physical and chemical procedures to *Brucella suis*, and by agar gel precipitation and absorption studies have identified a maximum of 6 antigens in the extracts obtained. The optimum conditions for release and the resistance of these antigens to heat and chemicals, including proteolytic enzymes, has been examined (p. 219).

WILHELM, HILL, ELIZABETH M. SPARROW, MARGARET E. MACKAY and MILES, continuing their work on a factor in serum which increases the permeability of small blood vessels (PF), its precursor (pro-PF), and its inhibitor (IPF), have shown that a pro-PF-IPF system exists in rat and rabbit blood. Rat PF appears in the α-globulin fraction and is like that of the guinea-pig in activity, and in being activated by dilution and inhibited by soya bean trypsin inhibitor (SBTI). It differs from guinea-pig PF by being inhibited by mepyramine. It is not a histamine or 5-hydroxytryptamine liberator, or plasmine. Rabbit PF occurs with both α and β globulin fractions, is not activable by simple dilution, and is insusceptible to mepyramine, SBTI, and guinea-pig IPF. Rat IPF occurs with the α_1-globulin and albumin fractions, and rabbit IPF migrates in the albumin zone (p. 228).

STONER has studied the effects at different environmental temperatures of limb ischaemia on the temperature of various organs and on liver blood flow in normal, acclimatized and adrenal medullectomized rats and on arterial blood pressure and O_2 saturation in the portal vein and aorta of normal rats. During and after limb ischaemia, liver and brain temperature fell. Arterial blood pressure, and O_2 saturation in the brain fell after the period of ischaemia, and liver blood flow fell some hours after 4 hr. of ischaemia. Total O_2 consumption fell after the removal of the tourniquets and this was not reversed by O_2 administration. In cold acclimatized rats, the same liver blood flow changes were seen but the liver and brain temperature was unaffected. Increase of environmental temperature increased the rate of fall of blood pressure and liver blood flow, but the liver temperature rose. At decreased environmental temperatures, the fall of tissue temperature was accentuated but there was no effect on the changes in liver blood flow (p. 251).

BURROWS and BACON selected mutants from a fully virulent strain of *Pasteurella pestis* with all permutations of the three virulence factors F1, VW and P. Living vaccines prepared from these mutants were used to actively immunize mice, guinea-pigs and rabbits. They found that in mice the highest protection against virulent challenge was given by F1 + VW + P +. In this species, VW+ appeared as important as the previously recognized important F1 in producing protection. In the guinea-pig VW+ detracted from the protective value of vaccines (p. 278).

HESS and PEARSE have used indoxyl substrates to study the properties of an organophosphorus resistant esterase of rat kidney. Histochemical and *in vitro* hydrolysis studies suggest that this enzyme system involves a cathepsin and cathepsin-like activity has also been shown in a number of other cells (p. 292).

unresponsiveness at a later age. We were unable to produce a lowered antibody response in 6-weeks-old chicks with a post-hatching dose that was equivalent to 87·0 mg. protein per kg. body weight, but a dosage of 523·0 mg. was very effective. A point of interest in regard to the lower dosage is that not only did it fail to produce an inhibitory effect on antibody response but also, as far as we could tell, it failed to sensitize the birds for a secondary type of response. If 6-weeks-old or older chickens were given this amount of antigen intravenously they would produce high titre antisera, and when re-injected after 1 to 2 months would respond with a secondary type response.

We were unable to determine the maximum length of the post-hatching period in which an unresponsiveness could be produced, though doubtless such a period can be established. When chicks were injected at 1, 2, 4, 8 and 12 days post-hatching and challenged at 6 or 8 weeks their antibody response was significantly lower than in controls of the same age. However, there was a difference in the length of the unresponsive period of the chicks that were injected with larger doses (based on body weight) of antigen. Only those injected on the first day showed a statistically significant reduction in antibody production when challenged at 12 weeks of age.

It is difficult with the evidence available to discuss what mechanism or mechanisms are responsible for a reduction in immune responsiveness to a soluble antigen in the chicken. The difficulty is enhanced when we try to compare the phenomenon with what occurs in the rabbit under similar conditions. In the latter animal the unresponsiveness seems to be permanent, while in the chicken it is transitory.

Campbell (1957), proposing a theory for antibody formation, postulated that " ... antigenicity is often, and perhaps always, intimately associated with incomplete intracellular fragmentation of material." He further states, " On the other hand, the attachment of a large intact foreign molecule to RNA might inhibit completely the participation of RNA in protein synthesis." We assume this would mean antibody synthesis. Since such large quantities of antigen were injected by us we thought it possible that " quantities " of this material were still in the animals' tissue and prevented antibody synthesis. We, therefore, injected a number of 3- to 4-weeks-old chicks, that had been injected within 24 hr. after hatching, with large quantities of a potent antiserum in order to " neutralize " any persistent antigen. This group of chickens, when challenged at 6 weeks, showed the same degree of unresponsiveness as the ones that were not given any antiserum. This experimental approach may, however, deserve further attention, perhaps with even larger doses of antiserum.

SUMMARY

A reduction of the chicken's immunological response to BSA can be produced with the injection of 22·5 mg. of BSA on the day of hatching.

A very large dose of BSA (135 mg.) given on the day of hatching will not bring about a complete or permanent suppression of the antibody response.

Injections of a large quantity of BSA in chicks up to 12 days of age produces a significant suppression of antibody formation when the birds are challenged at 6 and 8 weeks old.

The immunological unresponsiveness is transitory and its degree depends upon the size of the initial dose and the interval between this inoculation and the challenging injection.

Reduction in the unresponsiveness could not be changed by injecting large quantities of homologous antisera.

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SURVEY OF PAPERS

GREEN inoculated rabbits with splenic material from rabbits that had had courses of nitrogen mustard followed by simple intravenous inoculations of *Salmonella typhi* H cells at various times after the completion of the nitrogen mustard course. He shows that the recipient's response to the antigen is of the primary type if the splenic material comes from animals that either have not had a nitrogen mustard course or had finished the course 72 hr. or 96 hr. before the *Salm. typhi* inoculation. Animals that received material from donors who were inoculated with *Salm. typhi* 24 hr. or 48 hr. after the end of nitrogen mustard courses gave a secondary type of response (p. 213).

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VAN DEN BRENK has investigated the effect of histamine depletion on radiation lethality in rats. He shows that treatment with compound 48/80 caused increased radiation lethality. This effect was most marked in animals with chronic histamine depletion (p. 300).

MAY and WHALER have performed experiments on rats, mice and rabbits designed to show the site of absorption of *Clostridium botulinum* type A toxin from the alimentary tract. By feeding methods and by methods in which isolated portions of the gut were inoculated with toxin they have shown that greatest absorption occurred in the upper small intestine. They demonstrated toxin in the thoracic duct and they showed that cannulation of the intestinal or abdominal thoracic lymph vessels in rats gave partial or complete protection to animals fed toxin in their drinking water (p. 307).

BANG and JANET S. F. NIVEN report the cultivation of organized tissues for periods of 2–3 months and the results of preliminary studies on the infection of cultures of suitable tissues with a number of viruses, and with *Plasmodium vivax* (p. 317).

TEMPELIS, WOLFE and MUELLER inoculated chicks on the 15th or 18th day of incubation with Bovine serum albumin by the intravenous route. Subsequently they inoculated the birds in a similar manner with BSA at 6 or 12 weeks of age. They examined the serum of the animals on the 4th and 6th day after the second inoculation for the presence of antigen and antibody. They demonstrate suppression of the immunological response at 6 weeks in birds inoculated as 15-day embryos. This effect is transient and had disappeared by the 12th week (p. 323).

TEMPELIS, WOLFE and MUELLER inoculated chicks intraperitoneally with Bovine serum albumin during the first day after hatching. They show that it is necessary to give large doses of BSA at this time in order to produce suppression of the antibody response in later life. They show that the suppression of the antibody response is transitory and that it was not possible to produce complete or permanent suppression (p. 328).